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# BIOLOGICAL BULLETIN

## THE INFLUENCE OF MOLDS ON THE GROWTH OF LUMINOUS BACTERIA IN RELATION TO THE HYDROGEN ION CONCENTRATION, TOGETHER WITH THE DEVELOPMENT OF A SATISFACTORY CULTURE METHOD.

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At the Marine Biological Laboratory, Woods Hole, during August and September, 1927, it was observed that cultures of luminous bacteria (*Bacillus Fischeri, Beijerinck, Migula*) tended to deteriorate rapidly, the deterioration being progressive, so that finally subcultures were made daily, the luminescence becoming steadily less, and the culture was finally lost. A fresh culture was obtained by plating out luminous material of the same stock, recovered from an old Petri dish culture contaminated with mold. This culture grew vigorously for a time, and then degenerated. Since the bacteria in contact with the mold continued to grow and glow for some time, new cultures were isolated when necessary.

These bacteria were being used for physiological experimental material, and it was considered necessary to learn the reason for the deterioration of the cultures and devise a cultural method by which bacteria of the same strain could be maintained in vigorous condition throughout any given series of experiments. Luminous bacteria live normally in sea water, which is maintained constantly in a fairly definite alkaline pH range. They are considered to grow best on culture media of about the same pH value as the sea water. All of these cultures were grown on the same medium, supposedly of the proper pH, and it was sug-

gested by Professor Harvey that the trouble might be due to insufficient alkali reserve, the acid produced by the bacteria rapidly lowering the pH of the medium to a value unfavorable to their growth. The influence of the mold in causing continued light and growth might be due to alkali production. This explanation was favored by the result of pouring a solution of *M*/2 NaCl to which Clark's phosphate buffer, pH 8.0, had been added, over the surface of several Petri dish cultures which had ceased to glow. One, in which the light had been out only a few hours, again began to glow, and the luminesceice lasted for over eight hours. Others, in which the light had been extinct for longer periods, were not revived.

Friedberger and Doepner (1907) had studied the influence of various molds on the light intensity of cultures of luminous bacteria. They grew molds in bouillon, filtered the bouillon, and used this material in making up culture media. They found a greater intensity of light in cultures grown on these media than on controls prepared with ordinary bouillon. The one difference which they could establish between ordinary bouillon and bouillon in which mold had been grown was an increased alkalinity in the latter. Their figures show that 10 cc. normal bouillon neutralized .4 cc. *n*/10 NaOH to phenolphthalein, while 10 cc. of their "mold bouillon" neutralized .2 to .4 cc. *n*/10 $H_2SO_4$ to phenolphthalein. They arrived at the conclusion that the greater intensity of the light of cultures grown on "mold bouillon" was due in part to the increased alkalinity, and in part to "other properties" of the mold.

Molisch (1912) had shown that in general the intensity of light of cultures of luminous bacteria depended on the rate of growth. It is the opinion of the writer, for reasons given below, that the only cause for the increase in intensity of light and length of life of cultures of luminous bacteria grown in contact with mold is that of alkali production by the mold, which thus acts as an alkali reserve.

A series of experiments using solutions of *M*/2 NaCl plus Clark's phthalate, phosphate, and borate buffers, found to be nontoxic, showed that these bacteria glowed brightly in the pH range 5.7 to 8.7, the luminescence lasting for over an hour.

(Observations were not made after more than an hour had elapsed.) Below pH 5.2 the light lasted only a few seconds, above pH 9.0 for three minutes or less. The pH range in which growth can be expected lies then between 5.7 and 8.7, pH values outside this range being productive of rapid injury.

The culture medium in use was a peptone, beef-extract, glycerine agar, made up in sea water, the pH being adjusted to 8.2 with NaOH. As these bacteria live normally in an environment containing NaCl in about one half molecular concentration, favorable conditions are provided for the use of buffer mixtures. Molisch (1912) had shown that a number of salts other than NaCl might be used in culture media for luminus bacteria. A culture medium was made up in which one fifth mol of secondary potassium phosphate in 500 cc. distilled water was substituted for one half of the sea water. After sterilization the pH was adjusted with NaOH to 8.2. Separate lots of the same batch were colored with the Clark and Lubs selection of indicator dyes, covering the pH range from 1.2 to 9.8. Cultures were started on slants prepared from these media, six tubes of each being inoculated with luminous bacteria and three of each six being inoculated also with a common mold at one end of the slant. (The mold used was kindly identified for me by Dr. Charles Thom, as *Penicillium* sp., in the same section with *P. commune* (Thom) and *P. solitum* (Westling).) These were all allowed to develop somewhat below room temperature for two weeks.

Some of the indicators used were accumulated by the bacteria. These are being studied further to determine whether they penetrated the cell, or were merely adsorbed on the surface. They were of little value for this study, since not enough dye was left in the medium to indicate its pH value. However, in the case of several of these, the pH was indicated roughly by the color of the dead bacteria, which was not markedly different from the medium. With brom cresol green (yellow at 3.8, blue at 5.8), the dead bacteria near the mold were a more intense blue than elsewhere, and the acid range of the indicator had not been reached anywhere in the slant.

On the chlor-phenol red slants (yellow at 5.2, red at 6.8) the color of the medium indicated that the pH had been reduced to

5.4 $\mp$ .2. The pH of the medium near the mold was well above the alkaline range of the indicator.

On the cresol red slants (yellow at 7.2, red at 8.8) the color of the medium indicated pH below the range of the indicator except near the mold, where a pH of 8.6 $\mp$ .2 was indicated. The results with meta cresol purple were about the same. With thymol blue, the color of the indicator was masked by the color of the medium at the critical value, and it was of no value.

On the same date six cultures were started on medium of the same batch without addition of indicator. At the end of two weeks all were alive and glowing brightly. These cultures decreased slowly in brilliance during the next month, but were still glowing faintly at the end of six weeks, and viable transfers were made at the end of the seventh week. The final death of these cultures appeared to be caused by the drying up of the medium.

As a further check on the alkali influence, several cc. of *M*/NaOH was introduced at the bottom of each of six slants of unbuffered medium colored with brom thymol blue, and an equal number without indicator. Streaks made on these slants developed rapidly on the upper half of the slant, away from the alkali, and grew well, the cultures on the uncolored medium lasting for several weeks (average of six, 22.2 days), until the alkali was exhausted. On one of these, more alkali was added and a fresn inoculation made, the growth lasting this time for less than a week. It was observed that no growth took place below the line which marked the limit of diffusion of strongly alkaline NaOH. This limit was well marked on the uncolored medium by the precipitation of magnesium hydroxide.

The most characteristic activity of luminous bacteria seems to be that of acid production. They are killed in a few days in their own acid if some method of neutralization or removal is not employed. In their natural environment the excess acid would simply diffuse into the surrounding sea water, but within the limits of the test tube this cannot occur. The base used in the culture medium was NaOH, which in contact with carbon dioxide becomes $NaHCO_3$, and since $NaHCO_3$ in the concentration used (.01 *M*) is not particularly acid when saturated with carbon dioxide, it is not likely that the acid limiting their growth is car-

bon dioxide. That it is a non-volatile acid is shown by the following experiment:

A constant stream of sterile air was drawn in series through three bottles of slightly buffered culture medium colored with cresol red. The first of these was the control, without bacteria. The other two were inoculated with luminous bacteria. At the end of 24 hours the control was red, as at the start, and uncontaminated as shown by the absence of turbidity, and this condition lasted until the close of the experiment. The two inoculated bottles at the end of 24 hours were down to about pH 7.4. Enough NaOH was added to the third bottle to restore the original pH of approximately 8.2, and this was repeated every two hours until the close of the experiment. At the end of 36 hours, the PH in bottle No. 2 was down to about 5.5 (determined by withdrawing some of the material and testing with other indicators) and the light was extinct. In bottle No. 3, in which pH 8.2 was maintained, the bacteria continued to glow for another 24 hours, when the light failed, due presumably to failure of the food supply. Carbon dioxide and any other acids volatile at room temperature (if any were formed) would have been swept out by the stream of air, leaving behind the non-volatile acid. This is probably lactic acid.

Other culture media were tried in which calcium and barium carbonates were employed as buffers, and also higher concentrations of $K_2HPO_4$ and sea water, and lower concentrations. Luminous bacteria can tolerate a considerable range of salt concentration. It was found that on phosphate buffered media where the total salt concentration was greater than in sea water, but not in excess of molar concentration, growth was slower than on media of the proper concentration, and the tendency to diffuse growth was absent. The resultant crowding gave the streaks a fictitious brilliance for a few days, after which the light intensity decreased to a low value. These cultures were viable for fairly long periods of time, average 21 days, but not as long as cultures on media of the proper salt concentration. When media of lower total salt concentration (as about $\frac{1}{4}$ molar) were used, there was an initial rapid growth, accompanied by flowing over the surface of the medium, and a rapid decay, so that such cultures were

viable for only a few days, the average of six cultures being five days. Since a heavy precipitate of calcium and magnesium phosphates was formed when the phosphate buffer was added to sea water, media were prepared containing various concentrations of NaCl, from .25 *M* to .75 *M*, as substitutes for sea water, but these were unsatisfactory, the best of them lasting for only 14 days.

On medium buffered with calcium carbonate, growth was vigorous, but the life of the cultures was less than with the best of the phosphate buffer mixtures. The average length of life of eleven cultures without indicator was 17.8 days. Curiously enough, the death of these cultures was due to excess alkalinity. The initial growth was rapid, but on the third or fourth day there was a decrease in brilliance of light and a slowing down of growth, caused by the rapid diffusion of the acid through the agar, using up the small amount of calcium hydroxide in solution. This was followed by an increased brilliance and renewed growth as the pH rose again, due to the solution of more calcium hydroxide (produced by hydrolysis from the calcium carbonate), and its diffusion through the medium. The calcium salt of the acid produced by the bacteria is much more soluble than calcium carbonate, and is evidently hydrolyzed in solution, for the medium becomes steadily more alkaline until the alkaline range of the available indicators is passed. Since the bacteria are soon killed by alkali above pH 9.0, the limiting value is passed, and luminescence ceases. This can happen only when the calcium carbonate is in excess. When the pH of the medium was adjusted with calcium carbonate, and the excess carbonate filtered off, initial growth was rapid, but the decline following it continued until the death of the culture occurred on the sixth day (average of six cultures), caused by acidity as shown by the use of a suitable indicator.

On the medium prepared with barium carbonate from which the excess carbonate was filtered off, the initial fair growth was followed by a rapid decline, the average length of life of 14 such cultures being 6.5 days. When an excess of barium carbonate was present, the initial growth was fair, and slowly decreased, the cultures growing steadily more alkaline, the average length

of life of 14 cultures being 17.5 days. Although theoretically about the same pH value should be produced by barium and calcium carbonates, in practice the medium prepared with barium carbonate was always the more alkaline, and was too alkaline for good growth of the bacteria. The vigorous growth obtained on calcium carbonate was never obtained on media prepared with barium carbonate.

The medium prepared with calcium carbonate has the advantage that no adjustment of pH is required, the hydrolysis of the carbonate giving approximately the right value. It is by far the best buffer substance to use, both for slants and for Petri dish cultures. The medium should contain 20 grams "Bacto" nutrient agar, 10 cc. glycerine, and 5 grams calcium carbonate per liter, made up in sea water. If a transparent medium is desired, the phosphate buffer mixture with the same amount of nutrient substance, made up in sea water and filtered, may be used. The optimum pH value for this medium, probably about 8.6, may be secured by titrating the hot medium by the drop method until a good red is secured with cresol red, and a barely perceptible color with thymol blue. When one fifth mol of buffer is added to sea water, the average life of cultures emitting strong light is 18 days. After this time, very little light is emitted, but viable transfers may be made for several weeks.

Of the indicators used, several appeared to be slightly toxic to the bacteria, but the evidence on this point is inconclusive.

## Summary.

The influence of molds on the length of life of cultures of luminous bacteria may be simulated by the use of buffer mixtures, or by supplying fresh alkali continually. The maximum alkalinity produced in these experiments by the influence of *Penicillium* sp. was pH $8.6 \mp .2$. Degeneration of cultures of luminous bacteria may be caused by growth on media insufficiently alkaline, or so slightly buffered that it soon becomes acid. Diffuse growth and spreading over the surface of the slant is caused by too low salt concentration. Long life of cultures may be secured by growing on media sufficiently alkaline, and sufficiently buffered to resist rapid change by the acid production of the bacteria, which are killed by their own acid at about pH 5.6.

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# THE SEX RATIO IN *PEROMYSCUS*.

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The data herein presented are based on the records of breeding experiments with *Peromyscus*, conducted by Dr. F. B. Sumner. In an earlier paper [1] the sex ratio in *Peromyscus* was discussed at considerable length and data covering the years 1915–1921 inclusive were presented. The present report is based on the records of births from 1922–1926 inclusive. The material is made up partly of the various mutant strains of the *maniculatus* series, variously hybridized and partly of the three subspecies of *Peromyscus polionotus, P. p. polionotus, P. p. leucocephalus,* and *P. p. albifrons,* both pure and hybrid. No attempt will be made to give comprehensive interpretation of the findings but reference may be made to the paper cited above for more detailed discussion.

I take this opportunity of acknowledging my indebtedness and sincere thanks to Dr. F. B. Sumner who suggested the subject and under whose general guidance the work was carried out.

The influences which might affect the sex ratio in *Peromyscus* were considered in this treatment of the data to be (1) season, (2) size of litter, (3) race, (4) hybridization.

The total number of broods recorded in the records from 1922 to 1926 is 760, comprising 2,522 young, or an average of 3.32 mice per brood. According to sex these were distributed as follows:

| | |
|---|---|
| Males | 1,316 |
| Females | 1,114 |
| Sex undetermined (dead or escaped) | 61 |

The sex ratio (number of males per hundred females) for those of known sex is $114.93 \pm 3.19$.[2] It is interesting to note here that the sex ratio for the data from 1915 to 1922 was $97.37 \pm 1.93$.

[1] Sumner, McDaniel and Huestis, BIOL. BULL., No. 2, 1922.

[2] The probable error here employed is $\pm 67.45\ (1 + R)\ \sqrt{\frac{R}{n}}$, in which $R =$ sex ratio.

Since the number of individuals considered here is about half as great as that in the previous paper on *Peromyscus*, we shall present the data of this later period only for what they may be worth. At the suggestion of Dr. Sumner it was considered permissable to combine these additional data with the earlier records and thus, in a sense, bring some of the results on the sex ratio of *Peromyscus* up to date.

The total number of broods in the combined data from 1915 to 1926 is 2,321, comprising 7,547 young, or an average of 3.25 mice per brood. According to sex these were distributed as follows:

| | |
|---|---|
| Males | 3,597 |
| Females | 3,492 |
| Sex undetermined (dead or escaped) | 458 |

The sex ratio for the combined data is thus $103.01 \pm 1.64$.

## Season.

The following table gives the sex ratio for each month of the year and also the number of individuals upon which this ratio is based. The table contains the total data for the years 1922–1926.

| | |
|---|---|
| January (162) | $123.61 \pm 13.29$ |
| February (154) | $120.59 \pm 13.21$ |
| March (360) | $106.43 \pm 7.64$ |
| April (290) | $114.39 \pm 8.94$ |
| May (390) | $110.50 \pm 7.65$ |
| June (277) | $123.77 \pm 10.12$ |
| July (184) | $111.90 \pm 11.30$ |
| August (220) | $143.68 \pm 13.50$ |
| September (140) | $140.35 \pm 19.42$ |
| October (143) | $92.96 \pm 10.67$ |
| November (109) | $76.67 \pm 10.15$ |
| December (68) | $120.00 \pm 19.30$ |

As it is obvious from the graph that the differences between the consecutive months are of little significance we may combine our monthly birth records into four seasons of three months each. In both the earlier data alone and in the combined data we may distinguish two high periods and two low periods annually. The sex ratios for these four periods applied to the later data are as follows:

(1) February–April ..... 111.86 ± 5.43
(2) May–July ..... 114.99 ± 5.36
(3) August–October ..... 126.55 ± 7.77
(4) November–January ..... 105.56 ± 7.92

The greatest difference between two of these ratios is that between the third and fourth periods This difference is 20.99 ± 11.09.

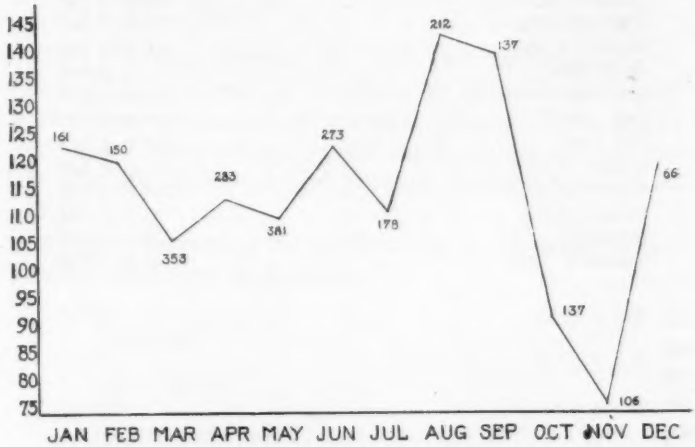


FIG. 1. The sex ratio of *Peromyscus* for each month of the year computed for the data from 1922 to 1926. The figures along the graphs denote the number of individuals born during each month of the year.

Grouping the same data according to the seasons of the year, as employed by King and some others, we get the following sex ratios:

Spring ..... 110.12 ± 4.90
(March–May)
Summer ..... 126.28 ± 6.71
(June–August)
Autumn ..... 102.13 ± 3.08
(September–November)
Winter ..... 121.76 ± 8.53
(December–February)

Here the greatest difference is between summer and autumn, being in this case 24.15 ± 7.38. Inasmuch as our figures are small we make no attempt to attach any particular significance

to these values but we may say in passing that they are of the same order of magnitude as the findings of King[1] in the Norway rat. In both we find a maximum in summer followed by a minimum in autumn.

Combining the earlier data (1915–1921) with these additional data we get the following monthly sex ratios:

| | |
|---|---|
| January (395) | 103.53 ± 7.41 |
| February (469) | 99.54 ± 6.17 |
| March (1,129) | 106.26 ± 4.30 |
| April (660) | 113.65 ± 6.20 |
| May (967) | 101.13 ± 4.61 |
| June (707) | 100.59 ± 5.29 |
| July (592) | 98.11 ± 5.74 |
| August (818) | 113.46 ± 5.46 |
| September (617) | 108.45 ± 6.03 |
| October (564) | 103.37 ± 6.02 |
| November (367) | 78.12 ± 5.76 |
| December (279) | 96.38 ± 7.93 |

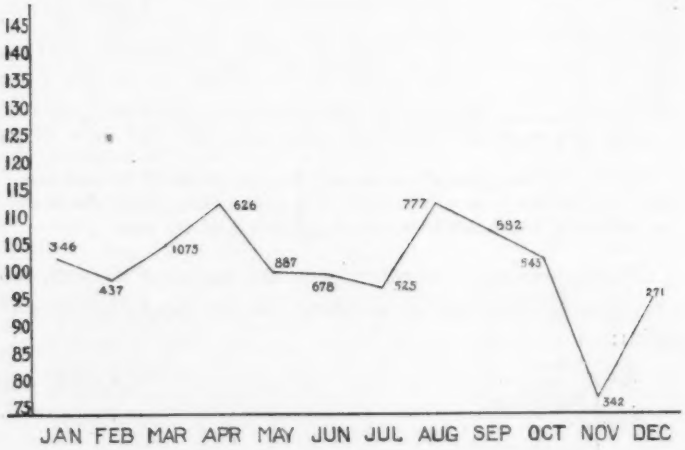


FIG. 2. The sex ratio of *Peromyscus* for each month of the year computed for the combined material from 1915 to 1926. Numbers along graphs indicate numbers born in each month.

Here, as in the earlier data alone, we find two annual maxima, one occurring in March and April; the other from August to Oc-

[1] *Archiv für Entwickungsmeckanik*, 1927, 61.

tober. In the graph we have the appearance of a fairly well marked biennial rhythm.

Now grouping the combined data according to 3-month periods we find the following sex ratios:

| | |
|---|---|
| (1) February–April | $106.96 \pm 3.07$ |
| (2) May–July | $100.19 \pm 2.96$ |
| (3) August–October | $107.87 \pm 3.36$ |
| (4) November–January | $91.80 \pm 4.01$ |

The difference between the third and fourth periods is $16.07 \pm 5.23$ and may be considered of probable significance according to the conventional statistical standard. These figures still show a rather marked biennial rhythm despite the fact that the later data showed reversed relations for the February–April period.

Again, if we regroup the combined data by the ordinarily recognized seasons the figures become:

| | |
|---|---|
| Spring (March–May) | $106.22 \pm 2.78$ |
| Summer (June–August) | $104.76 \pm 3.18$ |
| Autumn (September–November) | $97.43 \pm 3.45$ |
| Winter (December–February) | $100.00 \pm 4.18$ |

Here the greatest difference, between spring and autumn, is $8.79 \pm 4.43$ and of no probable significance. Likewise the biennial rhythm, apparent in the case of the later data seems to have been eliminated by the addition of the earlier data. This, we may say, is typical of the conflicting results pervading the entire literature on the sex ratio.

In the previous paper on the sex ratio in *Peromyscus* it was stated that the records were "unfortunately not adapted to revealing definite periods of increased or diminished reproductive activity, since the matings were to a large extent controlled in accordance with the demands of the breeding experiments." Since this statement is equally applicable to the later data, we wish to stress the point that only the number of matings was controlled and we cannot understand how this could possibly affect the normal seasonal trend of the sex ratio[1]

[1] *Cf.* King, 1927.

## Size of the Broods.

The mean size of the 760 broods considered in the later data is 3.32. The following table gives the sex ratios for mice belonging to broods containing from one to seven individuals respectively. Double broods or broods in which individuals of unknown sex are known to have died have been excluded.

| No. in Brood. | Males. | Females. | Ratio. |
|---|---|---|---|
| 1 | 17 | 12 | 141.67 ± 36.07 |
| 2 | 119 | 103 | 115.53 ± 10.44 |
| 3 | 423 | 336 | 125.89 ± 6.25 |
| 4 | 352 | 316 | 111.39 ± 5.83 |
| 5 | 141 | 129 | 109.30 ± 9.02 |
| 6 | 59 | 31 | 190.32 ± 28.36 |
| 7 | 18 | 17 | 105.88 ± 24.17 |

Summarizing the combined data we get the following table for the sex ratios according to the size of the brood:

| No. in Brood. | Males. | Females. | Ratio. |
|---|---|---|---|
| 1 | 81 | 73 | 110.96 ± 12.09 |
| 2 | 351 | 355 | 98.87 ± 4.96 |
| 3 | 1,047 | 993 | 105.44 ± 3.18 |
| 4 | 1,029 | 983 | 104.68 ± 3.18 |
| 5 | 405 | 385 | 105.19 ± 4.97 |
| 6 | 159 | 111 | 143.24 ± 11.96 |

Considering either the single or combined data we can find no significant differences in the sex ratios of various sized litters and we can only conclude that the size of the brood does not seem to have any well-defined relation with the sex ratio in *Peromyscus*.

Separate calculations were made for the litters in which no deaths were recorded and for the litters in which deaths are known to have occurred. In the later data we find the sex ratio for incomplete broods, comprising 43 broods, to be 83.64 ± 11.26. For the 673 complete broods the sex ratio is 118.09 ± 3.38—the difference between incomplete and complete broods being 34.45 ± 11.76. While this difference is large enough to be of interest we cannot attach any great significance to it inasmuch as only 43 incomplete broods were considered. In the combined data we find sex ratios of 91.45 ± 4.76 and 104.65 ± 1.79 based

on 309 and 1,974 broods for the incomplete and complete broods respectively. Thus we do find a difference between the sex ratios of complete and incomplete broods but we do not feel justified in regarding it as significant in view of the meagre record of identified dead.

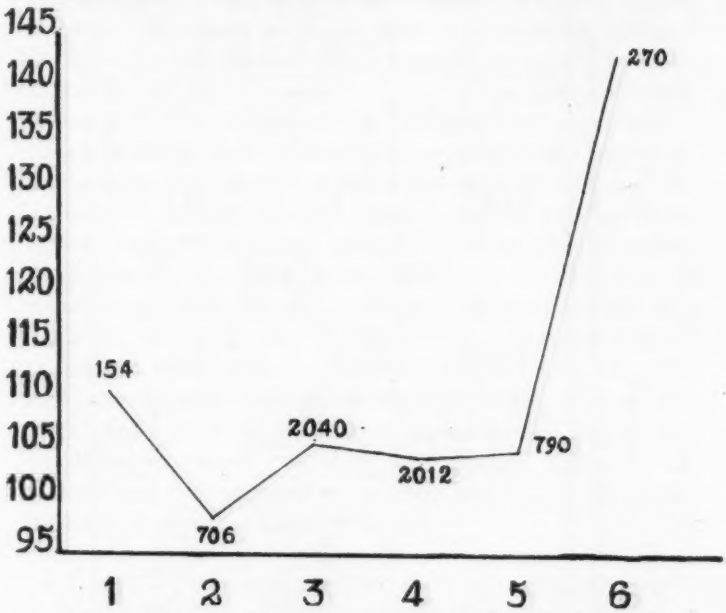


FIG. 3. Variations in the mean sex ratio, according to the size of the broods. Numbers along graphs indicate numbers of individuals.

*Combinations of the Sexes in Individual Broods.*

It is interesting to consider the possible tendency of members of a litter to agree with one another in respect to sex, that is, whether or not we encounter broods consisting entirely of the same sex more frequently than would result from chance. In the following table, using the combined data, we have arranged broods of each size in groups according to the number of each sex present. For example, broods of three present four possible combinations: 3 ♂, 2 ♂ + 1 ♀, 1 ♂ + 2 ♀, 3 ♀. The actual number of complete broods containing a given combination of males

## TABLE I.

NUMBER OF BROODS CONTAINING EACH POSSIBLE COMBINATION OF MALES AND FEMALES.

| Number in Brood. | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Combinations..... | | | | | | 1♂ | | 1♀ | | | | | |
| | No. of broods (actual) | | | | | | 81 | | 73 | | | | | |
| | Expected number.... | | | | | | 78 | | 76 | | | | | |
| 2 | Combinations..... | | | | | 2♂ | | 1♂, 1♀ | | 2♀ | | | | |
| | No. of broods (actual) | | | | | 83 | | 185 | | 85 | | | | |
| | Expected number.... | | | | | 91 | | 176 | | 86 | | | | |
| 3 | Combinations..... | | | | 3♂ | | 2♂, 1♀ | | 1♂, 2♀ | | 3♀ | | | |
| | No. of broods (actual) | | | | 92 | | 260 | | 251 | | 77 | | | |
| | Expected number.... | | | | 89 | | 259 | | 251 | | 81 | | | |
| 4 | Combinations..... | | | 4♂ | | 3♂, 1♀ | | 2♂, 2♀ | | 1♂, 3♀ | | 4♀ | | |
| | No. of broods (actual) | | | 30 | | 140 | | 183 | | 123 | | 27 | | |
| | Expected number.... | | | 33 | | 130 | | 188 | | 122 | | 30 | | |
| 5 | Combinations..... | | 5♂ | | 4♂, 1♀ | | 3♂, 2♀ | | 2♂, 3♀ | | 1♂, 4♀ | | 5♀ | |
| | No. of broods (actual) | | 8 | | 28 | | 45 | | 48 | | 22 | | 7 | |
| | Expected number.... | | 5 | | 26 | | 50 | | 49 | | 24 | | 5 | |
| 6 | Combinations..... | 6♂ | | 5♂, 1♀ | | 4♂, 2♀ | | 3♂, 3♀ | | 2♂, 4♀ | | 1♂, 5♀ | | 6♀ |
| | No. of broods (actual) | 0 | | 5 | | 16 | | 13 | | 8 | | 3 | | 0 |
| | Expected number.... | 1 | | 4 | | 11 | | 14 | | 10 | | 4 | | 1 |

and females and the "expected" number to the nearest integer are computed. Since the percentage of males in the combined data is 50.74, I have computed these last figures by expanding the binomial $(1.015 + .985)^n$. In the case of an equality ratio we should use the ordinary formula for probability, *e.g.*, $(1 + 1)^n$. Considering the comparatively small number of broods present in most of the groups we find a rather close agreement between the actual and the expected figures for all of the broods in which all members were of the same sex. The actual number of such homosexual litters, among broods containing from 2 to 6 individuals inclusive, was 409; while the most probable number on the assumption of purely random sex-production, was 422. If we consider fractions (a more exact procedure) this last figure becomes 420. In the earlier data alone a closer agreement than this was found, the figures being 276 and 274 for the actual and expected number of broods respectively. It would appear that the distribution of the sexes in single broods follows the laws of chance and there seems to be no tendency for fetuses (or germ cells) developing in the same parents at the same time to give rise to organisms of the same sex. We may likewise reiterate the conclusion of Sumner, McDaniel and Huestis, namely; "the non-occurrence of polyembrony or true twinning, at least with sufficient frequency to affect the results."

## Race.

For the later data we have computed the sex ratio separately for the "pure" (non-hybrid) polionotus series and in the following table we have listed in addition the sex ratios for some of the other geographic races (subspecies) as computed by Sumner, McDaniel and Huestis.

| Subspecies. | Males. | Females. | Ratio. |
|---|---|---|---|
| *polionotus* | 120 | 89 | 134.83 ± 13.15 |
| *gambeli* (La Jolla) | 770 | 840 | 91.67 ± 3.07 |
| *sonoriensis* | 350 | 373 | 93.83 ± 4.70 |
| *rubidus* | 150 | 124 | 120.97 ± 9.91 |

The difference between "*polionotus*" and "*gambeli*" is 43.16 ± 13.50 and may possibly be regarded as significant. But

we cannot say definitely that these figures imply the existence of any actual racial differences with regard to the sex ratio.

It is interesting to also observe here that in the subspecific hybrids of *Peromyscus polionotus* we find a sex ratio of $114.61 \pm 5.79$ while the subspecific hybrids considered in the earlier report (mainly *P. maniculatus*) give a mean sex ratio of only $104.76 \pm 3.41$.

Parkes[1] briefly summarizes the data of many workers on specific variations in the sex ratio in man and other mammals.

### Hybridization.

In 235 broods comprising 735 individuals of $F_1$ hybrids in the later series we find a sex ratio of $114.61 \pm 5.79$. While this is lower than the ratio for the pure "*polionotus*" stock (see p. 159), we cannot attach any significance to the latter figures since they are so small. For the same reason we do not feel justified in combining the later group with the earlier, in a comparison of pure and hybrid ratios. We may say, however, that in the earlier series alone the difference between the ratios for pure and hybrid stock was found to be $11.49 \pm 4.1$, the hybrid series giving the higher ratio. These results are in agreement with the conclusions reached by other workers, *c.g.*, Pearl (1908), King (1911), and Little (1919), that hybridization "per se" may result in raising the sex ratio.

### The Year.

The sex ratios and the number of individuals upon which they are based for the year 1922–1926 are as follows:

| | |
|---|---|
| 1922 (296) | $106.34 \pm 8.33$ |
| 1923 (355) | $106.43 \pm 7.64$ |
| 1924 (519) | $120.80 \pm 7.27$ |
| 1925 (966) | $113.93 \pm 5.03$ |
| 1926 (386) | $125.60 \pm 8.80$ |

Although it is quite evident that there are no significant differences here it was thought worth while to present the figures in view of the fact that the earlier data on *Peromyscus* (1915–1921) showed such marked yearly variations. While these results were

[1] A. S. Parkes, "The Mammalian Sex Ratio," *Biol. Review*, Vol. II., No. 1, Nov., 1926.

inexplicable, they were statistically speaking, the most significant of all and the likelihood of obtaining one of the differences by "accident" was less than one in 40,000. It was further proven that these differences were "not due either to the seasonal distribution of births, to the preponderance of hybrid births in one year as compared with another, or to the operation of any of the other factors previously considered."

Inasmuch as it is evidently exceedingly difficult to correlate the annual variation in the sex ratio with any known influences, accurate data on the subject are generally lacking. Of course it is not impossible that the most "significant" figures may result from chance.

## Summary.

Data have been presented based upon 2,522 deer mice as recorded during the breeding experiments of Dr. Sumner, from 1922 to 1926. Earlier records (1915–1921) were added to the above and the combined data have also been presented.

The following results seem to be of most importance.

1. The mean size of 760 broods in the later records is 3.32. For the combined data comprising 2,321 broods the mean size is 3.25 mice per brood.

2. The sex ratio for the later data is $114.93 \pm 3.19$; while that for the entire lot is $103.01 \pm 1.64$.

3. Considering the possibility of a seasonal cycle in the proportion of males and females born, we can only say that we find in the later data a maximum sex ratio in the August-September period followed by a minimum during October and November. In the combined data we find two annual maxima, one occurring in March and April, the other from August to October, and hence a fairly well marked biennial rhythm. Grouping the combined material according to 3-month periods we find in one arrangement that the biennial rhythm is practically eliminated while in another it is rather well marked. The existence of a seasonal cycle in the sex ratio of *Peromyscus* is not definitely proved.

4. The size of the brood in the combined material does not seem to have any well defined relation with the sex ratio in *Peromyscus*.

Although we find a difference between the sex ratio of complete

and incomplete broods we cannot regard it as significant in view of the meagre records of identified dead.

5. When the number of each possible combination of males and females, in broods of each size, is compared with the number expected according to chance, the conformity is found to be, on the whole, very close. For example, if we compare the actual and expected totals for all of the broods in which all members were of the same sex we find 409 as the actual number and 420, the "expected" number. Thus there is no preponderant tendency toward the production of homosexual litters and thus the non-occurrence of polyembrony or true twinning to any great extent.

6. While the sex ratio for the three subspecies of *polionotus* is "significantly" higher than that for other pure races of *Peromyscus* we cannot say definitely that these figures imply the existence of any actual racial differences with regard to the sex ratio in *Peromyscus*. The sex ratio of *polionotus* hybrids is likewise considerably higher than that of other *Peromyscus* hybrids which have been studied.

7. No significant yearly variations were found in the sex ratio of *Peromyscus* from 1922 to 1926.

# COLD HARDINESS IN THE JAPANESE BEETLE, *POPILLIA JAPONICA* NEWMAN.

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Cold hardiness, or the ability of an organism to withstand low temperature may be considered from two points of view, (1) cold hardiness to the intensity factor or the ability to survive extreme low temperatures, and (2) cold hardiness to the quantity factor or ability to withstand long periods of low temperature. By low temperature is meant, temperature below that required for normal development.

The Japanese beetle, which was introduced into the United States about 1916, can be secured in large numbers, thus making intensive study possible. This insect represents a type of ecological group, the soil dwelling insects. It passes the winter in the larval stage; about 97 per cent. in the third instar; about 3 per cent. in the second. Cold hardiness to both the quantity and intensity factor of low temperature was studied. Both external and internal factors are involved in cold hardiness. These include such environmental factors as relative humidity and temperature, and such physiological conditions as nutritional state, health, blood conductivity and metabolic rate. Most of the work was done on larvæ. Some studies were made on adults and a few observations were made on cold hardiness in pupæ.

## METHODS AND APPARATUS.

Respiratory rate and quotient were determined by the modified Krogh manometer of Bodine and Orr (1925). Conductivity of blood and body fluids was determined by the ionometer, described by Gram and Cullen (1923). pH was determined with the type K potentiometer, using a small vessel capable of testing the pH of a drop. By this method several readings could be taken on the same larva. This method was described by Bodine and Fink

(1925). Occasionally a larva was found that would not bleed freely enough to give sufficient blood for a reading. Blood was usually taken from one of the feet. Relative humidity was maintained by pulling air over different concentrations of sulfuric acid by means of a suction pump.

## Cold Hardiness to the Intensity Factor of Low Temperature.

In comparison with the oak borers previously studied by the author, Payne (1926), the Japanese beetles are less cold hardy and also exhibit less variation to low temperature. In Pennsylvania the most cold hardy Japanese beetle withstood — 28° C.; the most cold hardy oak borer —47° C. The most cold hardy Japanese beetle collected in the field thus far withstood — 15° C.

Periodicity in cold hardiness to the intensity factor of cold is not as marked in the Japanese beetle as in the oak borers *Synchroa punctata* and *Dendroides canadensis*. Comparison of the three species in question tested at the same dates is shown in Table I.

Conditions other than seasonal which modify the cold hardiness of the Japanese beetle to the intensity factor of low temperature are (1) degree of dehydration, (2) disease incidence, (3) nutritional state, and (4) temperature at which the larvæ were kept. Although these larvæ are seldom collected in dry places normally, they are able to withstand a high degree of dehydration. Larvæ dried down to a pulpy condition in which the free water is reduced to a minimum are cold hardy to both intensity and quantity factors of low temperature. Severe dehydration is accompanied by a high death rate. Larvae can be dried down to one third of their body weight. In the dehydrated condition the Japanese beetle larvæ reach their greatest cold hardiness. Since eighty per cent. of dehydrated larvæ die the effect of dehydration may be considered highly selective, killing off those larvæ unable to hold water. Those larvæ capable of resisting dehydration are cold hardy. Relative humidity affects cold hardiness in a decided manner. The results of a series of different experiments with varying relative humidities is shown in Table II.

TABLE I.

| Species. | September 29, 1926. | | | November 5, 1926. | | | December 11, 1926. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean Under-cooling. | $\sigma$. | C.V. | Mean Under-cooling. | $\sigma$. | C.V. | Mean Under-cooling. | $\sigma$. | C.V. |
| *Synchroa punctata* | −2.75 | ± .153 | .056 | − 7.5 | ±2.794 | .374 − | −12.4 | ±3.422 | .276 |
| *Dendroides canadensis* | −8.45 | ±2.64 | .313 | −15.6 | ±4.087 | .264 − | −17.34 | ±3.606 | .208 |
| *Popillia japonica* | −2.2 | ± .114 | .0518 | − 4.9 | ± .2156 | .044 | − 6.2 | ± .335 | .054 |

## TABLE II.

CONDUCTIVITY OF BLOOD OF JAPANESE BEETLE LARVÆ KEPT AT DIFFERENT TEMPERATURES.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

| 0° C. | 10° C. | 20–22° C. | 25° C. |
|---|---|---|---|
| .65 | .6 | .38 | .33 |
| .68 | .61 | .45 | .35 |
| .72 | .604 | .42 | .38 |
| .64 | .58 | .41 | .39 |
| .70 | .604 | .42 | .40 |
| .69 | .55 | .41 | .41 |
| .67 | .58 | .45 | .375 |
| .66 | .6 | .44 | .39 |
| .68 | .6 | .435 | .40 |
| .71 | | .46 | .42 |
| | | .45 | .39 |
| | | .43 | .41 |
| | | .445 | |

Starvation at high temperatures, 20° C. or above, is fatal to the larvæ unless the relative humidity is kept high. When kept at high humidity, larvæ are able to withstand comparatively long periods of starvation. One hundred larvæ were kept without food for the month of May, 1927, but under conditions of 100 per cent. relative humidity or saturation. Each larva was placed in an individual vial and weighed before and after the starvation period. During the process they lost about one half of their body weight. None of them survived freezing, the lowest freezing point was — 1.7°C.; the highest — .65° C. Larvæ kept at + 10°C., or below their developmental temperature, lost one half of their body weight. Starvation conditions were assured by keeping the larvæ in sterile white sand kept moist with distilled water. About one fourth of these larvæ survived freezing. Changes in body weight under different conditions of starvation and dehydration are shown in Fig. 1. The effect of prolonged exposure to low temperature as well as starvation was involved in the experiment described above. The effect of different temperatures on cold hardiness as measured by blood conductivity is shown in Table III. Larvæ starved for one week at + 20° C. increased in cold hardiness. In general early stages of starvation are marked by an increase in cold hardiness, later

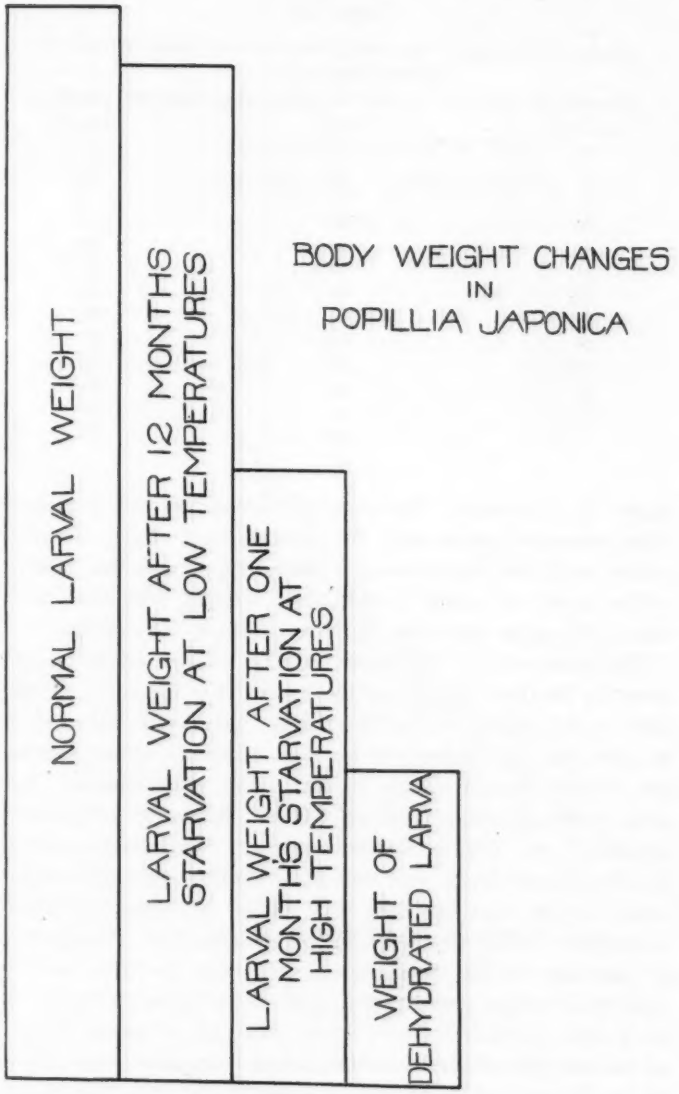
BODY WEIGHT CHANGES
IN
POPILLIA JAPONICA
NORMAL LARVAL WEIGHT
LARVAL WEIGHT AFTER 12 MONTHS STARVATION AT LOW TEMPERATURES
LARVAL WEIGHT AFTER ONE MONTHS STARVATION AT HIGH TEMPERATURES
WEIGHT OF DEHYDRATED LARVA

FIG. 1.

TABLE III.

EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON CONDUCTIVITY AT TEMPERATURE OF 22° C.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

| Saturation. | 80%. | 50%. |
|---|---|---|
| .33 | .46 | .72 |
| .38 | .45 | .75 |
| .35 | .47 | .76 |
| .39 | .465 | .78 |
| .40 | .44 | .73 |
| .41 | .455 | .77 |
| .375 | .46 | .74 |
| .39 | .44 | .76 |
| .40 | .455 | .77 |
| .375 | .46 | .76 |
| .38 | .45 | .82 |
| .32 | .46 | .77 |
| | .445 | .78 |
| | .43 | |

stages by a decrease. The point of decrease in cold hardiness from starvation comes when the digestive tract clears. In connection with this observation it is interesting to note that freshly molted larvæ are unable to withstand freezing until they have eaten. Pre-pupæ with clear digestive tracts are not cold hardy.

The occurrence of wilt disease in many of the specimens collected in the field offered an opportunity for the study of the effect of this disease on cold hardiness. Larvæ were collected at the same date and subjected to the same conditions of temperature and relative humidity, only healthy larvæ were studied. No larva showing typical symptoms of wilt disease or polyhedral-skränkheit was able to survive freezing. Since thermocouples used in diseased larvæ were difficult to sterilize and might infect healthy larvæ, cold hardiness was studied by measuring blood conductivity rather than freezing point depression. Conductivity decreases as the disease progresses. On the first day of apparent infection, conductivities of blood of diseased larvæ were below that of healthy larvæ. To produce such a marked change on the first day of infection, the causative organism must affect the blood very profoundly and very rapidly. On the other hand the change in conductivity may not be as rapid as it appears. The disease may be present in larvæ before it is detected by discol-

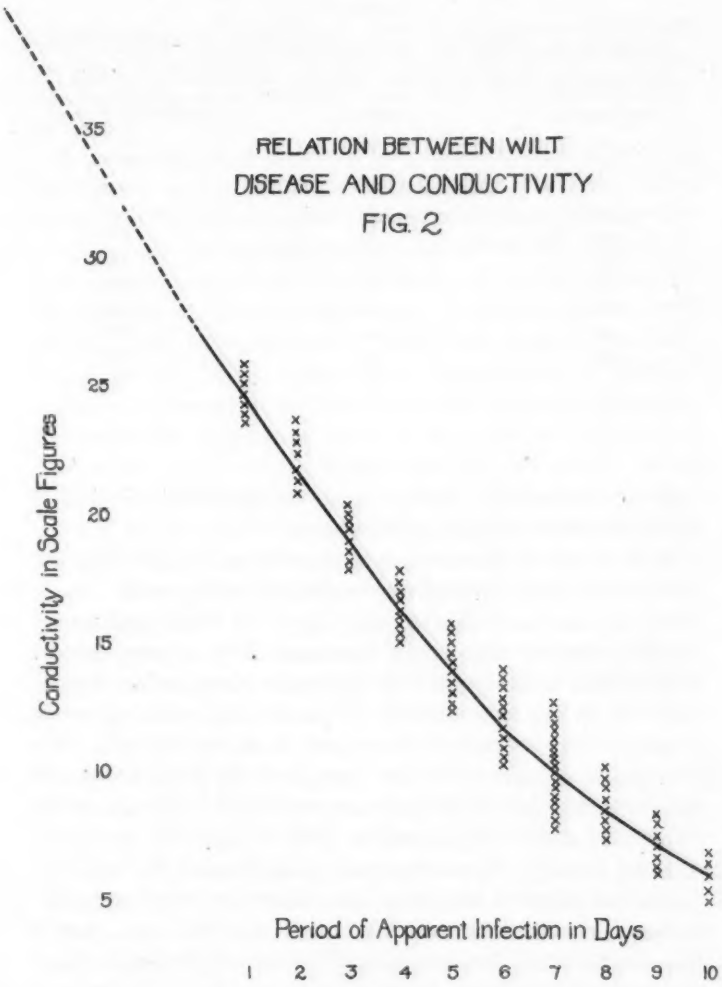


FIG. 2.

oration or wilting, and may be producing conductivity changes in the blood before other symptoms can be observed. A graph showing the relationship between day of apparent infection and blood conductivity is shown in Fig. 2. Table IV. shows the re-

## Table IV.

Conductivity of Healthy and Diseased Japanese Beetle Larvæ.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

| Wilt Disease. | Healthy. | Blackened by Freezing. |
|---|---|---|
| .21 | .38 | .6 |
| .17 | .4 | .6 |
| .10 | .41 | .604 |
| .11 | .4 | .604 |
| .04 | .39 | .58 |
| .05 | .375 | .575 |
| .07 | .43 | .61 |
| .18 | .425 | .63 |
| .12 | .41 | .62 |
| .06 | .39 | .625 |
| .13 | .4 | .64 |
| .25 | | |
| .27 | | |
| .15 | | |

sults of conductivity readings made on the blood of diseased larvæ in comparison with healthy ones.

Wilt disease is characterized by a pronounced blackening that precedes the final softening that occurs just before death. Blackening also has been observed when larvæ are frozen and thawed quickly. Blood from larvæ blackened after thawing always showed high conductivity. In these cases discoloration was believed to be due to changes in cell permeability releasing certain oxidative enzymes, which on escaping blackened the cells. The prothoracic segment is the first portion of the larvæ to discolor after freezing, both in the Japanese beetle and in the oak borers studied. Changes in permeability could be observed during the thawing process. Water apparently passes through the body wall where the chitin is thinnest. This water was frequently reabsorbed when the larvæ were kept under small bell jars. Larvæ losing water alone were generally able to survive freezing. When the fluid exhuding from the larva gave tests for amino-acids or proteins the larvæ always died. The exudate remained colorless for several days unless hydrogen peroxide was added, in which case it blackened quickly. Larvæ which showed the exudate after thawing were fixed and sectioned, but in these sections no gross differences from normal tissue could be detected. Broken

cell walls were not in evidence. The direct cause of death from extreme low temperature has been interpreted as due to an irreversible change in permeability rather than to a breaking of the cell walls.

If larvæ capable of surviving low temperature are ground up and filtered and the filtrate precipitated with lead acetate, there occurs in the filtrate an enzyme capable of breaking proteins down to amino acids at low temperatures and of building up proteins from amino acids at high temperatures. A similar enzyme has also been found in tussock moth eggs. Reversible reactions with proteases have been reported by Abderhalden (1914) from autolyzing tissues. Taylor (1909), found that a protein—"plastein"—could be formed from albuminose and proteolytic enzymes. The reversible reaction of starch to sugar at low temperatures and sugar to starch at high temperatures is a well known reaction that takes place in potato storage. The cold hardy mechanism of these larvæ studied may, in part, be due to enzyme action which transforms large protein molecules into smaller amino-acids. The larger number of osmotically active units thus formed would lower the freezing point.

Periodicity to cold hardiness is not as marked in the Japanese beetle as it is in some of the insects that are exposed to extremes of low temperature. Larvæ of the Japanese beetle live close enough to the surface of the ground to experience some seasonal change. During the spring and fall they are in addition subjected to diurnal temperature change. Cold hardiness in the larvæ appears to be closely related to their environment. These organisms are somewhat seasonal in their resistance to low temperatures. This periodic cold hardiness is shown in Table I. Comparison with oak borers and aquatic insects is shown more fully in a previous article by the author (Payne, 1926). Although the larva stage is the only one which overwinters in this climate, it was thought that studies on the cold hardiness of the adults would yield valuable material for the comparison of a stage exposed to winter conditions and a stage not normally exposed. Adults captured in summer and frozen without previous conditioning were able to survive ice formation within their tissues and to survive temperatures as low as — 20° C. Since it was im-

possible to obtain enough blood from the adults to make a conductivity reading none were made.

A beginning was made on the study of cold hardiness of the Japanese beetle pupæ. From present observations the age of the pupæ and consequently the degree of hydrolysis they are undergoing determines cold hardiness.

No changes in blood pH were found to be associated with cold hardiness in healthy larvæ. The pH obtained from a series of blood samples is shown in Table V. In the early stages of wilt

TABLE V.

PH OF JAPANESE BEETLE LARVÆ BLOOD THIRD INSTAR.

Each reading is an average of 3.

| Healthy. | With Wilt Disease. |
|---|---|
| 6.5 | 5.8 |
| 6.78 | 5.7 |
| 6.92 | 5.56 |
| 6.94 | 6. |
| 6.5 | 5.91 |
| 7.16 | 5.82 |
| 7.18 | 5.83 |
| 6.66 | 5.97 |
| 6.77 | 6.1 |
| 7.1 | 5.84 |
| 7.17 | 5.92 |
| 7. | 5.96 |
| 6.54 | 5.98 |
| 6.66 | 5.96 |
| 6.82 | 5.94 |
| 7. | 5.9 |
| 6.35 | 5.84 |
| 6.51 | 5.97 |
| 7.1 | 5.95 |
| 7.1 | |

disease the pH was lower than in healthy larvæ. In the late stages of the disease the larvæ were in such condition that it was difficult to obtain blood by cutting off the feet.

The respiratory quotient tends to be high in both cold hardy and non-cold hardy specimens, ranging from .67 to .72. The respiratory quotient of starving larvæ tended to be higher than well fed larvæ regardless of the temperature at which they were kept. The respiratory rate in larvæ in which cold hardiness had

been induced was much lower than in the non-cold hardy individuals. Associated with the low respiratory rate of hibernating forms was the slight change in body weight occurring over a period of several months, as shown in Fig. 1.

## Cold Hardiness to the Quantity Factor of Low Temperature.

Both the second and the third instars of the Japanese beetle larva are cold hardy to the quantity factor of low temperature except directly after molting or when the digestive tract is clear. Larvæ are markedly adapted to withstand long periods of low temperature. At the present writing there are still ten larvæ alive of one hundred which were placed at + 10° C. on December 6, 1925. These larvæ have now been kept over two years below their developmental temperature. Similar lots have been kept from six to twelve months at + 10° C. Graphs showing the number of larvæ surviving plotted against time in months in these experiments are shown in Fig. 3.

The relationship between survival for long periods at low temperatures and cold hardiness to the intensity factor of low temperature is shown in Table VI. The two types of cold hardi-

Table VI.

Survival after Freezing of Japanese Beetle Larvæ.

Kept at constant temperature of + 10° C. for varying periods of time.

| Length of Time Kept at + 10° C. | Number Frozen. | Number Survived. | % Survived. |
|---|---|---|---|
| 2 weeks | 1,450 | 1,426 | 98.34 |
| 4 weeks | 1,400 | 1,078 | 77 |
| 8 weeks | 1,000 | 645 | 64.5 |
| 3 months | 500 | 290 | 58 |
| 6 months | 200 | 48 | 24 |

ness appear to be inversely related after a certain point has been reached. This decrease in cold hardiness to the intensity factor of low temperature cannot be interpreted as a simple loss in vitality since larvæ kept at low temperatures are able to complete their development when placed at room temperature with no higher death rate than larvæ maintained at room temperature.

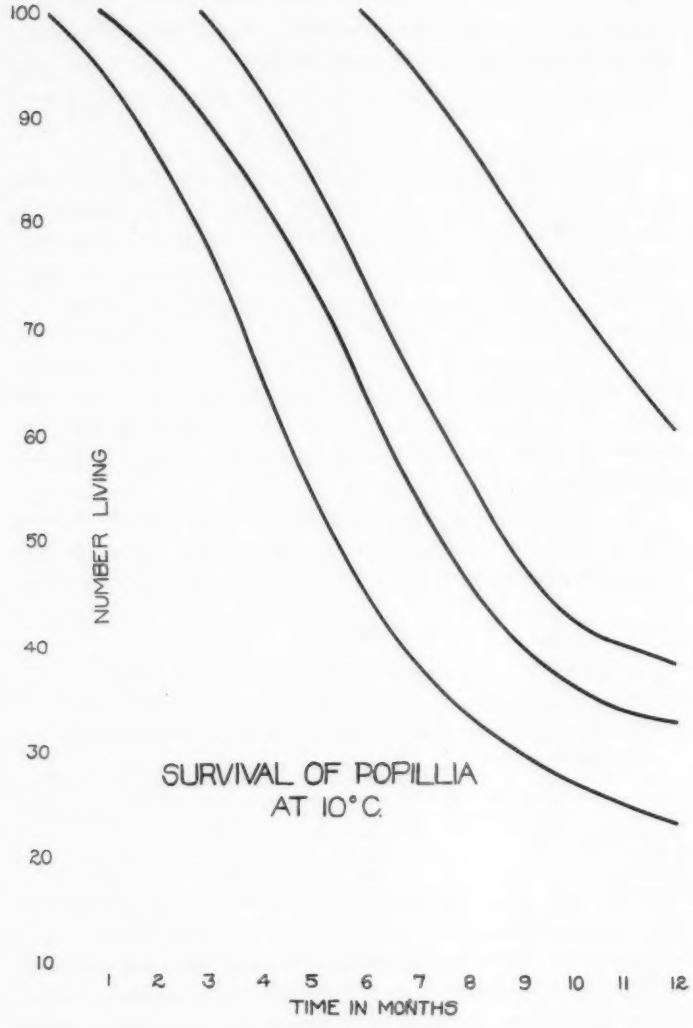
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
SURVIVAL OF POPILLIA
AT 10° C.
1 2 3 4 5 6 7 8 9 10 11 12
TIME IN MONTHS

FIG. 3.

Long periods of dormancy accelerate development when the larvæ kept at low temperatures are raised to developmental temperatures. Blood conductivity at first rises, then falls after two or more months when larvæ are placed at or below + 10° C.

The effect of rapid alternation between high and low temperatures on cold hardiness was tried with one hundred third instar larvæ. Temperatures of 0° C. and + 30° C. were alternated every twenty-four hours for one month. Neither of these temperatures is fatal. As controls one hundred larvæ were kept at 0° C. and one hundred at + 30° C. None died at + 30° C. Those alternated between + 30° C. and 0° C. died more rapidly than those kept at 0° C. Results of these experiments are shown in Fig. 4. In larvæ which had been exposed to wilt disease alternating temperature had no effect on length of life. None of these larvæ lived longer than ten days except when they were kept at or below 0° C. Healthy larvæ were considered exposed when they had been bitten by larvæ having wilt disease.

The respiratory quotient of larvæ cold hardy to the quantity factor of cold was somewhat variable but not connected to length of survival at low temperatures. In larvæ with clear digestive tracts it tended to become lower. In larvæ kept at + 10° C. it ranged from .69 to .73, or slightly higher than in larvæ cold hardy to the intensity factor of low temperature. In larvæ with clear digestive tracts low respiratory quotients were associated with lack of cold hardiness.

Low respiratory rate is associated with cold hardiness to the quantity factor of low temperature. Changes in body weight, as has been stated before, were very small with larvæ kept for long periods of time at + 10° C. These changes occurring in different states of nutrition and under varying temperature and humidity conditions are shown in Fig. 1.

Dehydration of larvæ is associated with cold hardiness to the quantity factor of low temperature as well as to the intensity factor of low temperature. Dehydration beyond two thirds of the body weight decreases cold hardiness to the quantity factor of low temperature. Over dehydrated larvæ lived but one day at 20° C. and not more than three days at + 10° C. or not more than four days at 0° C. Dehydrated larvæ have been kept for

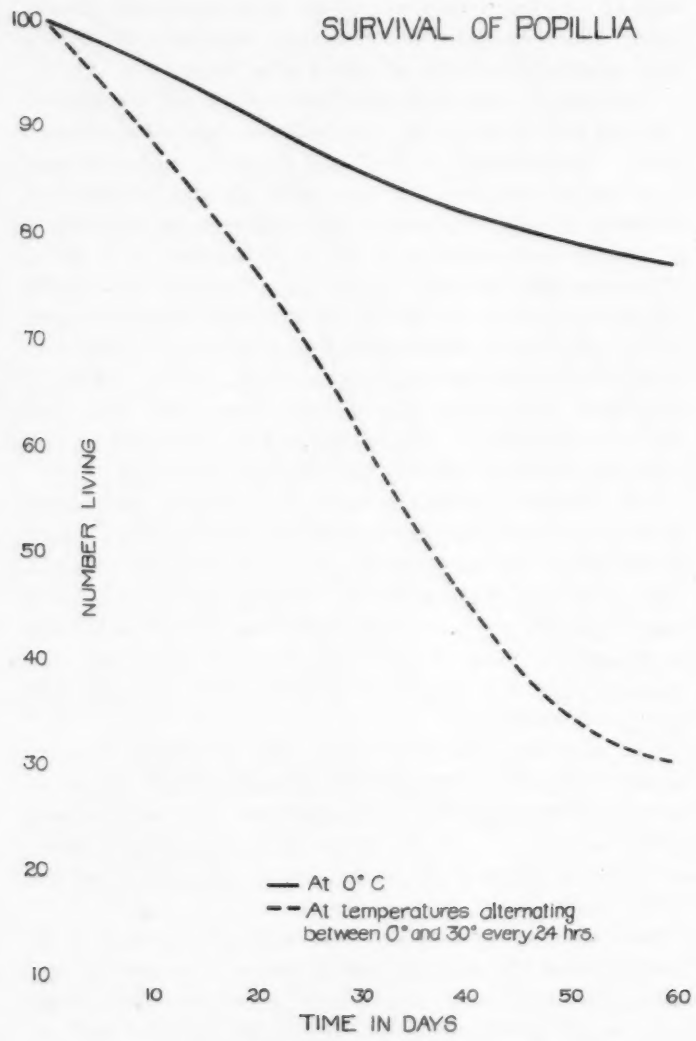
SURVIVAL OF POPILLIA
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
10
20
30
40
50
60
TIME IN DAYS
At 0° C
At temperatures alternating between 0° and 30° every 24 hrs.

FIG. 4.

one year at + 10° C. The experiment has not been continued long enough to determine whether or not dehydration increases the cold hardiness to the quantity factor of low temperature. Untreated larvæ are able to live two years or more below their developmental temperature. Dehydrated larvæ show very nearly the same death rate as undehydrated ones.

## Literature.

Since the literature pertaining to cold hardiness has been recently brought together it seems hardly necessary to make a detailed list and discussion of it. Robinson (1927) has discussed and given experimental data on water binding capacity as a factor in cold hardiness. Robinson (1926), and the author (1926) have summarized the literature. Hibernation in regard to both its ecology and physiology has been recently treated by Fink (1925), Townsend (1926), and Holmquist (1926).

From a survey of the literature it would appear that no one factor is an adequate measure of cold hardiness. The development of a cold hardy from a non-cold hardy insect is a deep-seated physiological process which affects blood, and body fluids, respiratory rate and permeability. Nutritional state and environmental conditions also influence cold hardiness.

## Acknowledgments.

The facilities for this study of cold hardiness were furnished by the Zoölogical Laboratory of the University of Pennsylvania. To Dr. J. H. Bodine, of the Department of Zoölogy, I owe thanks for many helpful suggestions, especially as regards apparatus and methods. To Dr. Henry Fox, of the Japanese Beetle Laboratory at Moorestown, New Jersey, I am indebted for most of the material used and for suggestions that have proved of service during the progress of the work.

## Summary.

1. Cold hardiness, both to the intensity factor and to the quantity factor of low temperature, were studied in the second and third instars of the Japanese beetle. Brief observations were made on pupæ and adults with regard to cold hardiness.

2. Japanese beetle larvæ are somewhat periodic in their cold hardiness to the intensity factor of low temperature, less so than the oak borers previously studied and more so than the aquatic insects.

3. Disease incidence, nutritional state, and degree of dehydration are associated with cold hardiness to the intensity factor of low temperature.

4. Development of cold hardiness to the quantity factor of low temperature is associated with loss of cold hardiness to the intensity factor except in extremely dehydrated individuals.

5. Marked permeability changes associated with enzyme action occur at the vital temperature minimum.

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# PELAGIC DISSOCONCHS OF THE COMMON MUSSEL, *MYTILUS EDULIS*, WITH OBSERVATIONS ON THE BEHAVIOR OF THE LARVÆ OF ALLIED GENERA.[1]

THURLOW C. NELSON.

The larvæ of the common black mussel, *Mytilus edulis*, are abundant in plankton samples taken throughout most of the summer in all regions where this mollusc occurs, Stafford, '12. Recognition of the larva, as Stafford points out, is rendered easy owing to its horn yellow color, its relatively small umbones and its small depth. To these characteristics may be added the distinctive shape of the shell, being more pointed and of shorter height at the anterior end, Fig. 1. The size of the mature prodissoconch when ready to attach varies considerably as judged from measurements of the largest larvæ obtained from the plankton, and from measurements of the prodissoconch shell of newly attached dissoconchs. Measurements of ten of the largest larvæ found in the plankton in Maine waters are as follows, antero-posterior axis being given first.

| | |
|---|---|
| 360 × 338 μ, | 368 × 320 μ, |
| 360 × 320 μ, | 336 × 304 μ, |
| 376 × 344 μ, | 360 × 312 μ, |
| 350 × 312 μ, | 360 × 320 μ, exclusive of dissoconch rim, |
| 336 × 304 μ, | 304 × 280 μ, exclusive of dissoconch rim. |

The last two larvæ, although caught in the plankton, each bore a narrow rim of purple dissoconch shell, Jackson, '88. From these and from other measurements made upon *Mytilus* larvæ it appears that dissoconch shell may be secreted at any time after the larvæ attain a length between approximately 300 and 360 μ. Stafford, *l.c.*, gives the measurements of two mature prodissoconchs as 345 × 310 μ and 400 × 331 μ.

[1] From the Zoölogical Laboratory of Rutgers University. Paper No. 14, New Jersey Oyster Investigation Laboratory.

The observations here reported were made during August, 1924 and August, 1927, in Frenchman Bay, Mt. Desert Island, Maine.[1] A collecting station some 100 meters from the labora-

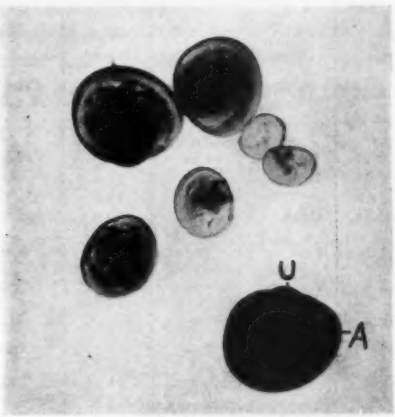


FIG. 1. Stages in the development of the prodissoconch larva of *Mytilus edulis*: *U*, umbones; *A*, anterior end.

tory point was marked with a buoy. With approximately 12 meters depth at mean low water this station lay in the full sweep of the tide through Frenchman Bay. Plankton samples of 25 liters were pumped here from various depths using a hose and oscillating clock pump, the majority of the samples being taken at the surface and at 7 meters depth. The *Mytilus* larvæ were collected by passing the water through a No. 18 treble extra heavy bolting cloth net, adding two or three drops of formalin to the catch and then drawing off the supernatant water bearing great quantities of the diatoms *Chætoceros* and *Rhizosolenia*. Table I. gives the numbers and stages of development of the mussel larvæ taken at the station, together with other data.

[1] It is a pleasure to acknowledge my indebtedness to the former Director, Professor Ulric Dahlgren, for the facilities given me at the Mount Desert Island Marine Biological Laboratory at Salisbury Cove, and for making early summer plankton catches for me.

TABLE I.

WATER CONDITIONS AND NUMBERS OF *Mytilus* LARVÆ AT STATION OFF LABORATORY POINT, FRENCHMAN BAY, MT. DESERT ISLAND IN 1924.

| Date. | Time. | Tide. | Depth. | Temperature ° C. | Mytilus Larvæ in 25 Liters. | |
|---|---|---|---|---|---|---|
| | | | | | Prodisso-conch. | Disso-conch. |
| Aug. 1. | — | — | Towing | — | — | 1 |
| 5. | 11:30 A.M. | 2/3 flood | 2 m. | 10.6 | Many | 1 |
| 7. | 3:00 P.M. | High | 7 m. | 11.0 | 262 | |
| | | | 0 | 13.3 | 753 | |
| 8. | 3:45 P.M. | High | 7 m. | 10.9 | 336 | |
| | | | 0 | 17.1 | 71 | |
| 9. | 11:30 A.M. | Low | 7 m. | 11.9 | 278 | 2 |
| | | | 0 | 15.6 | 5 | |
| 11. | 10:15 A.M. | 1/2 ebb | 7 m. | 13.7 | 63 | |
| | | | 0 | 14.3 | 6 | |
| 11. | 10:30 A.M. | 1/2 ebb | Towing | 14.3 | Many | 8 |
| 12. | 10:10 A.M. | 1/3 ebb | 7 m. | 11.1 | 1,500 | |
| | | | 0 | 13.9 | 4 | |
| 13. | 11:00 A.M. | 1/6 ebb | 7 m. | 11.7 | 650 | |
| | | | 0 | 13.8 | 6 | |
| 13. | 2:50 P.M. | 3/4 ebb | 7 m. | 11.7 | 213 | |
| | | | 0 | 15.3 | 20 | |
| 14. | 3:30 P.M. | 1/6 flood | 7 m. | 12.3 | 240 | 1 |
| | | | 0 | 12.9 | 320 | |
| 15. | 10:45 A.M. | High | 7 m. | 11.0 | 390 | 7 |
| | | | 0 | 12.9 | 1 | |
| 16. | 11:20 A.M. | 5/6 flood | 7 m. | 11.0 | 1 | 2 |
| | | | 0 | 12.9 | 177 | |
| 18. | 10:50 A.M. | 2/3 flood | 7 m. | 10.8 | 65 | |
| | | | 0 | 15.9 | 1 | |
| 19. | 3:10 P.M. | High | 7 m. | 12.2 | 152 | |
| | | | 0 | 13.2 | 2 | |
| 20. | 11:30 A.M. | 1/3 flood | 7 m. | 11.9 | 56 | |
| | | | 0 | 12.7 | 2 | |
| 21. | 2:50 P.M. | 5/6 flood | 7 m. | 11.6 | 30 | |
| | | | 0 | 12.0 | 4 | |
| 22. | 5:00 P.M. | High | 7 m. | 11.4 | 24 | 1 |
| | | | 0 | 14.7 | 5 | |
| 23. | 3:10 P.M. | 2/3 flood | 7 m. | 11.0 | 17 | |
| | | | 0 | 13.7 | 7 | |
| 25. | 3:00 P.M. | 1/3 flood | 7 m. | 11.7 | 40 | 1 |
| | | | 0 | 13.1 | 10 | |
| 27. | 3:45 P.M. | 1/6 flood | 7 m. | 12.3 | 19 | |
| | | | 0 | 13.9 | 7 | |

## PELAGIC DISSOCONCHS OF *Mytilus*.

### (*a*) *Buoyancy through Gas Secretion.*

In the tow sample taken by Professor Dahlgren August 1 was found one *Mytilus* larvæ which bore the distinct rim of purple shell which marks the commencement of the dissoconch stage.

Since only one such mussel was found it was believed to have been accidentally introduced through the townet striking some object bearing attached mussels. Subsequent collections, however, revealed numerous dissoconchs up to 941 $\mu$ in length freely floating about at various depths up to 7 meters. A 25 liter sample pumped August 13, from the surface, 20 cm. from a *Mytilus*-covered pile, yielded 200 *Mytilus* larvæ from mature prodis-

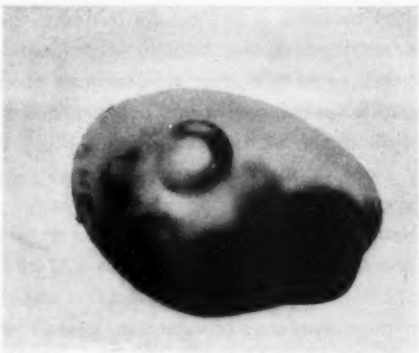

FIG. 2. Pelagic dissoconch of *Mytilus edulis* approximately .8 mm. in length, bearing a large bubble of secreted gas within the branchial chamber. This specimen came from a depth of 7 meters.

soconchs to advanced dissoconchs over 900 $\mu$ in length. A similar sample pumped August 15 from a depth of 7 meters at the collecting station, more than 100 meters from the nearest mussel beds, gave 390 prodissoconch *Mytilus* and 7 dissoconchs which ranged in length from 445 to 784 $\mu$.

The presence of well-developed dissoconchs floating freely in the water at once raises the question of the means by which this is effected in the absence of the swimming organ or velum of the prodissoconch. When brought to the laboratory for examination these dissoconchs were found to be identical with others removed from sea weeds, save for the presence of a large clear space in the posterior portion of the pallial cavity. Believing that some change might have occurred in the molluscs even during the fifteen minutes to half an hour which elapsed between their capture and subsequent examination in the laboratory, a

binoular was taken in the boat and the young mussels were examined immediately after their capture. The result is shown in Fig. 2. A large bubble was found to occupy the posterior part of the pallial cavity, its buoyancy causing the young bivalve to hang suspended in the water umbone downward, with the postero-ventral margin of the valves turned upward. On one occasion the bubble was seen to form through the coalescing of many minute bubbles, which, passing slowly down between the gill filaments, united to form a single large bubble. In several individuals two or three smaller bubbles were found. Where a single bubble was present its size was such as to cause a thinning of the mantle on either side and a forward displacement of the posterior gill filaments, thus accounting for the large clear space already noted in the posterior pallial cavity of the young *Mytilus* dissoconchs first taken.

Failure to observe the bubble in larvæ first brought to the laboratory was due to the fact that as soon as a *Mytilus* dissoconch comes in contact with any object the foot is rapidly extruded from between the valves and brought into contact with the surface. The extrusion of the foot, accompanied as it is by a separation of the valves and of the applied lobes of the mantle, results in the immediate escape of the bubble in nearly every instance.

The composition of the gas in the bubble was not determined owing to its small size and lack of adequate facilities for a microchemical test. The fact that it forms within the gills would indicate that it is mainly oxygen. The composition of gas secreted into the swim bladders of fishes renders this still more probable.

The possibility that the bubbles within the branchial chamber of these young *Mytilus* might have been air introduced accidentally during passage through the pump or while in the net, was tested in the following manner. The hose was disconnected from the pump and allowed to siphon water from a depth of 7 meters into the net held in the bottom of the boat. The stream entered the net under water and great care was taken not to agitate the net or to break the water surface. Dissoconchs of *Mytilus* collected in this way revealed the same large bubbles as before. Dissoconchs of *Mytilus* collected from sea weeds and

violently shaken with a little sea water in a bottle failed to acquire any bubbles of air in the process; thus, with the above experiment, proving that the bubbles of gas were not accidentally introduced.

To determine the possible effects of pressure in bringing about gas secretion pieces of glass tubing 2 cm. long were cut and an early *Mytilus* dissoconch obtained from sea weeds was introduced into each. A piece of coarse bolting cloth was tied over each end of the tubes which were then fastened to a line at one meter intervals and suspended from a float at the collecting station. One string bore seven tubes which hung at depths of from two to eight meters. A second string was attached to a weight on the bottom with a float of sufficient size to hold the string vertically in the water, the lowest tube being at 11 meters depth at low water and approximately 15–16 meters at high water. When removed 48 hours later all of the bivalves were found to have attached by the byssus to the inside of the tubes or to the bolting cloth ends. When removed to a dish of sea water they crawled actively about with the foot. In no case was a bubble present. Either the stimulus to gas secretion is absent when the mussels are attached, or the frequent extrusion of the foot which occurs while the mussel is attached permits the escape of such gas as rapidly as it is formed.

### (*b*) *Attachment to the Surface Film.*

If the surface of the water near a mussel bed or near a mussel covered piling be skimmed with a plankton net during the latter part of the breeding season, numerous dissoconchs will be found. They are most numerous as the rising tide first sweeps over the mussels and attached sea weeds. A microscopic examination of these dissoconchs shows that none contains a bubble, hence it is obvious that these young mussels must maintain themselves at the surface through means other than the gas secretion employed by larvæ at a depth. When placed in a dish of sea water such larval mussels exhibit great activity, gliding about upon the long, highly adhesive, ciliated foot as rapidly as a snail. Observations were made upon these young molluscs using a chamber 0.5 cm. wide made of two microscopic slides, filled with sea water and viewed horizontally through the binocular.

Once in contact with a solid object, such as the wall of the chamber, a rock, or a fragment of sea weed, the mussels exhibited a marked negative geotropism and climbed straight upward until the surface was reached. Here the distal one third to one tenth of the foot was extended in the surface film, Fig. 3, and with a quick contraction of the foot, aided apparently by contraction also of the pedal retractor muscles, the ventral margins of

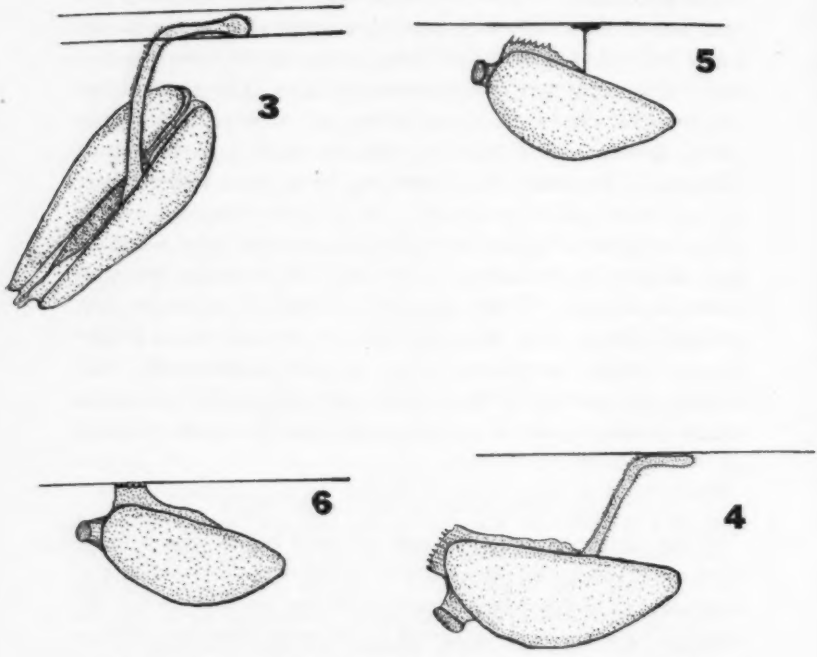


FIG. 3. Ventral view of *Mytilus* dissoconch, 4 mm. long, hanging from the foot in the surface film, as seen from the side and partly from above.

FIG. 4. Lateral view of 4 mm. *Mytilus* dissoconch hanging from the surface film. The siphons are fully extended.

FIG. 5. Lateral view of 3 mm. *Mytilus* dissoconch hanging from byssus thread attached to holdfast secreted in the surface film. The foot, which was fully extended in the surface film while secretion of the holdfast was effected, has been wholly withdrawn between the valves.

FIG. 6. Lateral view of 3 mm. *Mytilus* dissoconch holding to the surface film with the aid of the tentacles of the incurrent siphon. The foot which serves to hold the mussel close to the film until the siphon is inserted therein, has been withdrawn between the valves.

the mantle were brought into contact with the surface film. While lying with the entire ventral margin of the body in contact with the surface film the byssus gland in a few seconds secreted onto the surface film a small holdfast similar in appearance to that which is laid down on rock or piling for the attachment of each byssus thread. A thread 1–2 mm. long serves to support the young mussel from this float and with foot withdrawn it may hang suspended indefinitely, Fig. 5. At times it thrashes about with the foot fully extended as though in search of some solid surface for attachment. When the foot strikes such an object the mussel glides quickly upon it, trailing the byssus thread behind or breaking it off. The "float" is not a buoyant structure, since when pushed beneath the surface film it rapidly sinks. It maintains its position in the surface film, supporting mussels up to 4 mm. in length, solely through surface tension.

A float and connecting thread are not always secreted when the young mussel reaches the surface. At times it supports itself solely by the distal end of the foot in the surface film, Fig. 4, after the manner described for the prodissoconch oyster larva, Nelson, '24a. With the aid of the numerous short cilia covering the foot the animal glides slowly along the surface film, rocking the body slowly from side to side and occasionally through a quick contraction of the proximal portion of the foot, bringing the entire ventral margin of the shell in contact with the surface film. This behavior will recall the familiar habit of pond snails of hanging from the entire foot spread out in the surface film.

A third mode of suspension from the surface consists in extending the tips of the tentacles of the incurrent siphon into the film and hanging from these, Fig. 6. This behavior, though seldom observed, serves to support the mussel quite as effectively as does the foot.

Such floating dissoconchs have never been found further than approximately 25 meters distance from mussel beds or mussel covered piling. Their abundance, 5 to 100 per 25 liters of water, close to such habitats indicates that young mussels frequently make use of this mode of transportation for covering short distances.

Examination with the low power binocular of several small

tide pools close to the laboratory revealed numbers of *Mytilus* 3–4 mm. long moving actively over the rocks and barnacles while others were at the surface. With the incoming tide the latter are carried away and may eventually reach a place of attachment at a considerable distance.

### Metamorphosis in Allied Lamellibranchs.

The water samples taken in Frenchman Bay contained in addition to the larvæ of *Mytilus edulis*, great numbers of the young of the soft clam, *Mya arenaria*, together with occasional specimens of the larvæ of *Venericardium*, of *Anomia* and possibly also of *Astarte*. During more than ten years study of the oyster larvæ of the New Jersey Coast I have become familiar also with the larvæ of *Mytilus recurvus* (the southern oyster mussel), *Venus mercenaria*, and *Teredo navalis*. In no instance have I ever observed gas secretion in any of these forms nor have I found pelagic dissoconchs. When the time for setting arrives the mature larvæ of all of the above species disappear from the water within 24 to 36 hours.

Reproduction and dispersal of marine pelecypod molluscs occur through the medium of pelagic veligers which are free-swimming for periods ranging from a few days in such incubatory forms as *Ostrea edulis* and *Teredo bartschi*, to approximately three weeks in *Mytilus edulis*, *Mya arenaria*, and in probably most of the marine bivalve molluscs which reproduce at temperatures below 20° C., Nelson, '28. Owing to the sessile or sedentary habits of the adult molluscs, the activities of the larvæ become of first importance in the dissemination and preservation of the species. Through the aid of the velum the larval bivalve, while unable to make progress against a current, can control its vertical distribution and thus secondarily may determine to a marked degree its horizontal distribution by tides and currents, Nelson, '22.

The rate of growth and of development during larval life is determined chiefly by the temperature. The long series of observations on the life history of the American oyster, *Ostrea virginica* Gmelin (J. Nelson, Stafford, Churchill, T. Nelson and others), indicates that at a given temperature the duration of the pelagic period is remarkably constant. With an average tem-

perature of 23–24° C. the period from spawning to the attachment of the spat in New Jersey waters is 13 days. In Richmond Bay, Canada, J. Nelson, '17, found that at temperatures approximating 20° C. the minimum time required for oyster larvæ to mature was 17 days. Stafford, '13, considers three weeks to be the average time required to reach maturity in Canadian waters.

The close of the free-swimming period of pelecypod larvæ is determined apparently by internal developmental factors: when the time for "setting" arrives the larvæ must attach or die. Since the veligers during their pelagic existence have been distributed widely by currents it follows that for those which through chance happen to "fall upon good ground" there will be many more which through this same chance will "fall by the wayside" and be destroyed.

Observations of the oyster larva, T. Nelson, '22, '24, show that approximately 24 hours prior to attachment the young bivalve becomes positively stereotropic and that it may explore numerous surfaces with the aid of the foot before it finally attaches. Such behavior, while of the utmost importance in securing a favorable spot for attachment, is without avail if no substrata suitable for attachment are present. Little is known of the factors necessary to provide a favorable bedding ground for such burrowing species as *Mya* and *Venus*. Although attachment of young *Mya* by the byssus to sea weeds or other objects may occur, as shown by Ryder, '89, and by Kellogg, '99, it is pointed out by Belding, '12, that survival of both *Mya* and *Venus* depends largely upon the character of the mud and sand forming the surface layers of the bottom. All who have studied the habits of larval bivalves agree that the vicissitudes of larval life and subsequent attachment form one of the chief barriers to wide dispersal of the species.[1]

[1] A survey of our present knowledge of the habits and life histories of both fresh-water and marine pelecypods shows that of all environmental influences the presence of a suitable substratum is the most important single factor limiting distribution. The following papers may be cited in this connection: fresh water mussels, Coker et al., '21; *Mya*, Belding, '09; *Pecten*, Belding, '10; *Venus*, Belding, '12; *Ostrea cucullata*, *O. angasi*, Roughley, '25; *Mytilus*, *Cardium*, *Saxidomus*, *Siliqua*, *Paphia*, and other genera of the Pacific Coast, Thompson, '13, and Weymouth, '20; *Ostrea*

Of the known genera of marine pelecypods, *Mytilus edulis* and *Teredo navalis* alone are circumpolar in their distribution over the shores of the northern hemisphere. General adaptability to changing conditions and the power to resist adverse surroundings together with relatively low spawning temperatures, Nelson, '28, have aided these two forms in attaining their present wide distribution. Transportation through attachment to vessels or to other floating wood has likewise aided in their dispersal, being for *Teredo* the only means by which any great distance could be covered. In the case of *Mytilus edulis*, however, the ability to bridge the period of metamorphosis while remaining pelagic must have been an important factor in securing the wide dispersal which this mollusc now enjoys; as well as a great aid in bringing to a suitable place of attachment a fair proportion of the larvæ produced each season. The largest of the pelagic dissoconchs found in Frenchman Bay was fully a month old, during which time it must have been transported over long distances by the tide. If during this period it had come in contact at any time with a solid object attachment could easily have been effected.

## Summary.

At the close of the larval or prodissoconch stage the young of *Mytilus edulis* which fail to secure immediate attachment may remain pelagic through the secretion of gas into the mantle chamber.

Short distances may be covered through the aid of a holdfast secreted on the surface film or through holding the foot or the tentacles of the incurrent siphon in the surface film.

The ability to bridge over the critical period of metamorphosis while remaining pelagic has been an important factor in securing the present wide distribution of the black mussel.

Absence of a similar adaptation in the larvæ of other bivalves renders them still dependent largely upon chance in securing at the close of the pelagic period a proper substratum for attachment. This dependence upon the substratum is one of the chief barriers to the wide dispersal of the species.

*virginica*, Moore, '97, Grave, '01, Stafford, '13, J. Nelson, '17, Churchill, '20, T. C. Nelson, '22.

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# STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

## VI. A FEMALE OF *CAMBARUS IMMUNIS* WITH OVIDUCTS ATTACHED TO OPENINGS OF SPERM DUCTS.

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The specimen to be described was taken from Root River near Racine, Wisconsin, on July 10, 1924.

The presence of a normal annulus ventralis and of the usual rudimentary appendages upon the first abdominal segments mark

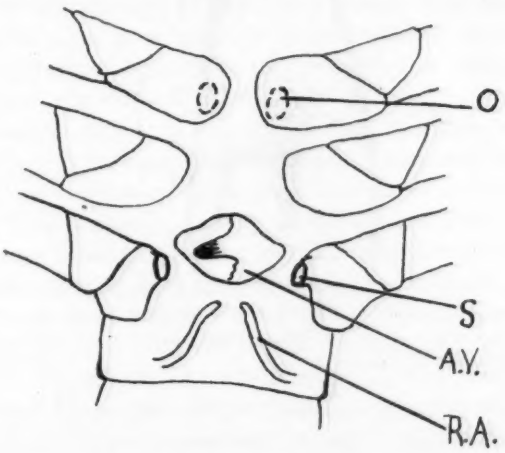


FIG. 1. Diagram illustrating external secondary sexual characters. *S*, openings of oviducts at base of fifth walking leg. *A.V.*, annulus ventralis. *R.A.*, rudimentary first abdominal appendages. *O*, position at base of third walking leg of oviducal pore in normal female.

it as a female but there are no oviducal pores at the bases of the third walking legs. On the other hand, a pair of openings appears at the bases of the fifth walking legs at the position ordi-

narily occupied by the openings of the sperm ducts. No other male characters are found, however (Fig. 1).

Upon dissection the specimen was found to have a well-developed ovary and oviducts which were attached to the ovary at the usual point. In normal specimens the oviducts make their way laterally and ventrally and open through pores located at the bases of the third legs. In this case, however, the oviducts slope posteriorly as well and finally attach themselves to the openings at the bases of the fifth walking legs. A closer examination of the pores themselves shows that they are essentially the openings of sperm ducts in structure although they are considerably modified (Fig. 2).

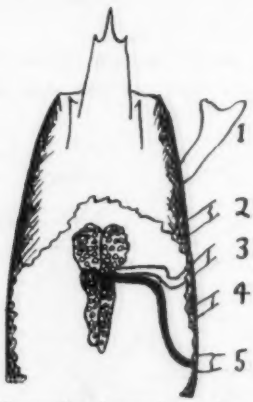


FIG. 2. Diagram illustrating internal relations of ovary, oviduct and walking legs. Position of oviduct in this specimen shown in solid black. Position of oviducts in normal female shown by lines. Positions of walking legs indicated by numerals.

The normal oviducal pore is oval in outline and has slightly raised and thickened rims, but the structure, as a whole, is not raised conspicuously above the surface of the shell. The greatest width of the oviducal pore in a specimen of this size is about one mm. with the length a little more. The plane of each pore is tilted a little toward the median line and a little toward the rear.

The normal openings of sperm ducts are much smaller and are extended by means of membranous projections. They open toward the median line and there is practically no deflection ventrally or posteriorly.

The openings of the specimen described here resemble the pores of sperm ducts in their general position, in the direction in which they open and in being projected somewhat by membranes. On the other hand, they are much larger than the openings of sperm ducts but smaller than normal oviducal pores and in shape resemble oviducal pores.

## Discussion.

Several questions of interest arise in connection with this case but most of them can be treated only as speculations. (1) How does it happen that the oviducal pores are absent in a female animal? (2) How does it happen that the openings of sperm ducts are present in a female animal? (3) Is the duct to be interpreted as an oviduct which has become attached at its distal end to the opening of a sperm duct or as a vas deferens which has become attached at its proximal end to an ovary? (4) To what extent has there been a modification during its development of the pore of the sperm duct by virtue of its attachment to the oviduct? (5) To what extent does the oviduct possess the potentiality of shaping a structure to which it is not ordinarily attached (the opening of the sperm duct) in the direction of an organ to which it is usually attached (the oviducal pore)?

As regards the first and second questions, it is being repeatedly shown in crayfishes that the contemporary occurrence in an animal of the ovary or spermary and a fixed set of secondary characters is by no means necessary for normal functioning. Indeed it has been shown that in some localities the occurrence of a charter supposed to be fixed upon one sex occurs upon the other sex considerably more than half of the time. The lack of one or both oviducal pores in otherwise normal females has also been recorded. The absence of oviducal pores is not surprising, therefore, nor the presence of the openings of sperm ducts. The presence of the openings of sperm ducts in the absence of oviducal pores does not mean that these are mutually exclusive structures, for oviducal pores are sometimes found upon males normal with respect to the sperm duct openings as well as otherwise normal.

The tube connecting the pore at the base of the fifth walking legs and the ovary may be considered an oviduct for the follow-

ing reasons. First, it is straight and shows none of the coils of the vas deferens. Second, it is thin walled and wide while the vas deferens is rather narrow and dense in texture. Third, it exhibits the same types and arrangement of tissues as a normal oviduct.

In *Cambarus* the only misplaced oviducal pores discovered have occurred on the second or the fourth walking legs of females and then only as supernumerary pores, the normal oviducal pores being present on the third walking leg as usual. It is assumed, therefore, that the pores of the fifth walking legs are the openings of sperm ducts and not of oviducts. The extent to which the pores depart from the features of the normal sperm duct is taken to represent the extent of the influence of the attachment of the pores to oviduct instead of sperm duct.

There is no evidence to show that the influence of the oviduct is required in embryology for the proper shaping of the oviducal pore. Rather to the contrary the oviducal pores are sometimes present and perfectly developed in the absence of oviducts. At the same time the resemblance of the pores of this specimen to oviducal pores must have been due to the influence of the oviduct to which they were attached. The case is roughly parallel to the results of the experiment in which an optic cup in a vertebrate embryo was transplanted under ectoderm in another part of the body and under the influence of the optic cup the ectoderm developed a lens although normally it would not have done so.

The condition described here is not to be confused with the normal condition found in some South American species belonging to the genus *Parastacus*. In these there are regularly both oviducts and sperm ducts but only one set is functional, that one being appropriate to the sex in which it occurs. The case described in this paper cannot be considered as a parallel, for here only one set of tubes is present and only one set of external pores.

# STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

## VII. REGENERATION OF ABERRANT SECONDARY SEXUAL CHARACTERS.

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It is rather generally conceded that intersexuality in insects has as a background either genetic disturbances or metabolic interferences due to parasitism. The evidence for the causes of intersexuality in the Crustacea, however, is by no means as distinct, due to the fact that it has not been possible to separate the question of the development of secondary sex characters from that of their control by hormones located in the gonads. It has been rather concisely demonstrated in the insects that secondary sex characters are independent of spermatic or ovarian hormones, whereas some of the evidence obtained in the Crustacea seems to indicate the possibility of such hormone control.

Among some six or seven causes which might be able to account for intersexuality among the decapods there is the repeated suggestion that some ambiguous or accidental event in embryology has been responsible. When in one locality eighty-six per cent. of all the females of *Cambarus virilis* and in another locality thirty-seven per cent. of *Cambarus propinquus* possess well-defined male characters, it would seem that no accidental event in embryology could account for this unusual occurrence. Rather, it would seem necessary to seek for some orderly and fixed influence. It occurred to the writer that the tendency to form aberrant structures might be tested for its duration beyond the embryonic stage by detaching the appendages bearing these structures and determining whether there would then be developed normal or aberrant structures. The accumulation of this body of data and the interpretation of it has been carried out with this inquiry in mind.

## Regeneration of Normal Secondary Sex Characters.

When any appendage is lost in the crayfish, unless the crayfish is too old, a new appendage will grow in place of the old one and if the crayfish is young when the accident occurs the regenerated appendage will come to have almost completely the size and characteristics which the original appendage would have had. In an old specimen, however, there will not be sufficient time for the complete regeneration of the appendage before the crayfish dies.

Some of the external secondary structures which have to do with sex are either modified appendages or structures located upon the appendages. Such are the first and the second abdominal appendages which are modified for copulation in the male but are rudimentary in the female, the hooks located upon the third walking leg of the male, and the openings of the oviducts and sperm ducts upon the third and the fifth walking legs respectively. When one of these appendages is broken off so as to include one of the modified structures, the appendage will begin to regenerate, beginning with the first moult. At first it is juvenile in character and unmodified but eventually it becomes completely differentiated and contains the modified structure. The regenerated secondary sex characters never completely resemble the normal secondary sex characters. In the case of the hooks which are found upon the third walking legs, the regenerated ones are blunt and flatter than the original ones, but occurring as they do in a definite position they are easily recognizable.

## Observations on the Regeneration of Aberrant Secondary Sex Characters.

Not more than thirty cases have been recorded in *Cambarus* of male-like modifications of the abdominal appendages in females and the possibility of finding cases in which the aberrant appendages have been injured and regenerated would be very remote. The occurrence of female structures upon males is also too rare to give any expectation of finding regenerated aberrant structures. The copulatory hooks upon the third legs have been selected, therefore, as the most likely structures for observation because their occurrence upon females furnishes the most common aberration.

In Lake Delavan (Wisconsin) eighty-six per cent. of the females of *Cambarus virilis* carry the copulatory hooks like those which occur upon the third legs of the males. It is as fully developed in the female as it is in the normal male and developes in ontogeny at the same stage. In the Menomonee River (Wisconsin) thirty-seven per cent. of the females of *Cambarus propinquus* also bear these hooks. These two localities were chosen as most likely to produce the desired specimens.

In the course of three summers collecting after several thousands of specimens had been examined, seven males and three females of *Cambarus virilis* from Lake Delavan were found, each of which had lost and regenerated one of the third legs. In each specimen the third leg had regenerated to a point where it was possible to determine whether or not a normal copulatory hook was being formed. Males, ranging from 56 to 88 mm. in length, had all somewhat imperfectly developed new hooks upon the regenerated legs (Fig. 1). These normal males with regenerated hooks

FIG. 1. Diagram illustrating three basal segments of third walking legs and copulatory hooks in male crayfish which has lost and regenerated a part of left leg containing copulatory hook. Regenerated copulatory hook is short and blunt.

were used as controls with which to compare the females which had likewise lost their third walking legs and had regenerated the third legs together with the hooks upon them. Of the females, two had well developed hooks upon the uninjured third legs and upon the regenerated third legs the hooks had reformed as in the males (Fig. 2). In the third female the uninjured third leg carried no hook and upon the regenerated third leg no hook had formed.

In the Menomonee River, two specimens of *Cambarus propinquus* were found which could be used for this study. The first was a male in which the uninjured third leg was entirely normal and carried the usual hooks. The left third leg had been lost at

an early stage and had regenerated. Upon it was the blunt type of hook usually found upon the regenerated third leg. The second was a female which also carried a hook upon the uninjured third

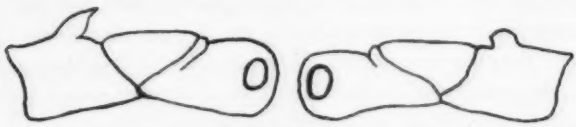

FIG. 2. Diagram illustrating three basal segments of third walking legs in an aberrant female which had lost and regenerated a part of the third walking leg. The copulatory hooks are aberrant and the left one has been regenerated.

leg and had also developed a blunt hook upon the regenerated third leg.

## EXPERIMENTS.

Young specimens of *Cambarus virilis*, about thirty-six mm. in length, were selected for experiment. They were taken from Lake Delavan on July 17. Eighty-two males in which the copulatory hooks on the third legs were visible were divided into two equal lots. One lot was used as a control and in the other, one of the third legs was detached in each specimen. Fifty-five females in which a copulatory hook was visible upon the third leg were divided into two lots and similarly operated upon or used as controls. All were kept in the laboratory under conditions as nearly natural as possible for ten months and about one fourth of the specimens survived. Six of these were males in which one third leg had been removed and upon the regenerated leg there had developed the copulatory hook. Seven were females from which one third leg had been removed. All such females had regenerated the third legs together with the copulatory hooks upon them. The hooks compared favorably with those regenerated by the males of approximately the same age.

## CONCLUSIONS.

In all the cases cited above, whether observed in nature or experimentally produced, females bearing aberrant male hooks upon their third walking legs regenerated hooks whenever an injured leg had sufficiently developed. Some were one year of age and

others were older. It is reasonable to state, therefore, that all the evidence, though meager, tends to show that whatever influence was present in the first place to produce this aberrant secondary sex character was also present and operative in the animal later during any regeneration period. The permanency of this influence during the life of an animal would seem to take it out of the classification of accidental or temporary embryonic agencies. When it is considered together with the fact that the same aberrancy is repeated in this crayfish population (observed for six years) it seems logical to give the influence a genetic status and to postulate that there has been a definite change within the germ cell.

# REGENERATION OF *LUMBRICULUS* IN VARIOUS RINGER FLUIDS.

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## INTRODUCTION.

In the course of work with Ringer solution on *Planaria*, J. W. Wilson ('26) has noticed that wound closure may be more or less completely suppressed in an isotonic solution. With the intention of making use of this peculiarity if it held true for *Lumbriculus*, I have experimented with various strengths of Ringer solution on this form. Finding various modifications of the usual method of wound closure and regeneration, I have made studies on the effects of various strengths of Ringer fluid on regeneration in this form. It is my purpose to report these at this time.

As a preliminary, the approximate osmotic pressure of the body fluids of this worm were determined in order that it might be possible to know something concerning the relative strengths of the internal fluids and the external solutions used. Adolph ('25, p. 332) concludes that we can "probably regard the maximum survival concentration for freshwater animals as a measure of the osmotic pressure of their body fluids." The maximum survival concentration of Ringer solution for *Lumbriculus* at the end of 24 hours (the arbitrary time adopted by Adolph, '25, for Phagocata) was found to be 0.147M. When corrected for ionization this gives a figure of 0.257M as compared with 0.215M for *Lumbricus*, as assumed by Adolph and Adolph ('25). Apparently a Ringer solution of between 0.14M and 0.15M concentration is approximately isosmotic with the body fluids of *Lumbriculus*.

## WOUND CLOSURE IN VARIOUS CONCENTRATIONS OF RINGER SOLUTION.

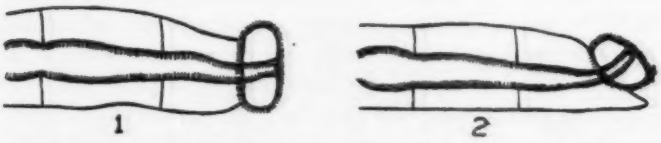
The usual method of wound closure in microdrilous annelids has been quite completely described (von Wagner, '00 and '06,

Iwanow, '03 and Krecker, '10, among others), both from the gross and from the microscopical points of view. Briefly it takes place in somewhat the following manner: Immediately after a worm is cut the muscles of the body wall begin to contract. They continue to draw the cut edges of the hypodermis together until only a small opening is left. This aperture is then clogged by a plug of cells, many of which have been torn free by the cut. The wound is thus completely closed and the body fluids once more prevented from freely mixing with the solution in which the worm is cut. At the same time the intestine also contracts somewhat, closing over at the end and withdrawing slightly from the contracting body wall. This preliminary wound closure is completed in from 10 to 15 minutes after the cut is made.

The behavior in an isotonic solution is in distinct contrast to this usual behavior. The following description of what occurred in one series of observations might well apply to many cases which have been followed for considerable periods.

An individual is cut in a 0.14M Ringer solution, without anaesthetization, at 2:54 P.M. Both pieces move about quite rapidly at first but in 3 or 4 minutes they have quieted down to ordinary "crawling" movements, such as are commonly found in uninjured individuals. During this time there is a loss of some blood and a number of cells due to the fact that there is no semblance of contraction of the body wall. At 3:03 there is evidence of a protrusion of the gut beyond the end of the body wall. This protrusion of the gut gradually becomes more pronounced until a portion, perhaps a segment in length, extends beyond the plane of the cut at 3:10. At this time the cut end of the gut begins to show evidence of a rolling back upon itself. This process continues until at the end of an hour there is a well formed bulb of everted gut present at the cut end (Fig. 1). During this time there has been a gradual contraction of the body wall until it has reduced the diameter of the opening resulting from the cut to about two thirds of its original size. The gut in the course of its eversion has now come in contact with the body wall so that there is very little opportunity for interchange of materials between the body fluids and the external solution. This is the end of wound closure from the macroscopical point of view.

As a result of this process of "wound closure" there is present at the cut surface, at the end of 1 or 2 hours, a bulb of everted gut usually of almost as great diameter as that of the body. The ciliated portion of the gut cells are thus exposed to the outside solution, in which they continue to beat with apparently the usual rapidity.




FIGS. 1 AND 2. Bulbs of everted gut at the posterior end of pieces regenerating in 0.091M Ringer solution. Fig. 1 after 12 hours; Fig. 2 after 6 days.

In the case of slightly hypotonic solutions the resulting bulb of gut is usually smaller, in some instances not more than one fourth the diameter of the worm. The presence of these smaller bulbs is due, in part at least, to the fact that in these cases the gut does not protrude as far at first, so that when the eversion occurs there is only a short piece involved. A contributing factor is the slight pulling together of the body wall; this probably tends to hinder the protrusion of the gut and in addition reduces somewhat the diameter of the portion which does pass through the aperture.

POSTERIOR REGENERATION IN VARIOUS RINGER SOLUTIONS.

If we assume that the maximum survival concentration at the end of a twenty-four-hour period is isotonic with the body fluids of an animal, it is difficult to keep individuals in an isotonic fluid for a very long period. One experiment may be cited to show what occurs when individuals are left for a long period in 0.147M Ringer, the solution being changed each day at the time of observation. Fifteen worms were put into such a solution and on the following day all were alive, with no ill effects apparent. On the second day, 2 had died and 3 others were clearly not far short of death. On the third day, 4 more were dead and 4 others were beginning to disintegrate at the posterior end. On the fifth day, only 5 were alive and 1 of these was beginning to disintegrate

at the posterior end. Because of this high mortality in Ringer stronger than 0.14M, it has been found advisable to observe the regenerative processes in the latter concentration in which the majority of the regenerating head ends will live for a considerable period. Even in this strength many tails die in the course of 3 or 4 days.

In the case of heads, regenerating new tails, which have been cut and left in 0.14M Ringer, many individuals have bulbs very similar to those found after 2 or 3 hours, even at the end of a week. For example, of 12 heads put into such a solution, 9 had bulbs of everted gut, 1 had a short and very slender bud of regenerating tissue and 2 had died at the end of a week. This proportion of individuals with bulbs of gut holds true within fairly narrow limits in all experiments using 0.14M Ringer.

In light of these results obtained with a solution almost isotonic with the body fluids, it is of interest to determine the effect of slightly greater dilutions of Ringer solution. The number of bulbs of gut present on individuals "regenerating" in various strengths of Ringer solution for 7 days is given in Table I.

TABLE I.

NUMBER OF INDIVIDUALS WITH BULBS OF GUT IN DIFFERENT CONCENTRATIONS OF RINGER SOLUTION.

10 worms were cut in each concentration.
Figures in parenthesis indicate number which also show new tissue.

| Molar conc. of Ringer. | Days of Regeneration. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 0.011 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.023 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.034 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.045 | 3(1) | 1(1) | 1(1) | 1(1) | 1(1) | 1(1) | 1(1) |
| 0.057 | 2(2) | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.068 | 3(1) | 3(2) | 1(1) | 1(1) | 1(1) | 1(1) | 1(1) |
| 0.079 | 8(2) | 8(4) | 8(7) | 8(8) | 8(8) | 8(8) | 8(8) |
| 0.091 | 7(3) | 7(4) | 7(7) | 7(7) | 7(7) | 7(7) | 7(7) |
| 0.102 | 8(1) | 8(5) | 8(8) | 8(8) | 8(8) | 8(8) | 8(8) |
| 0.113 | 10(0) | 10(0) | 10(1) | 10(4) | 10(10) | 10(10) | 10(10) |
| 0.125 | 9(0) | 9(0) | 9(0) | 9(3) | 9(5) | 9(6) | 9(6) |
| 0.136 | 10(0) | 10(0) | 10(0) | 10(0) | 10(0) | 9(0) [1] | 9(0) |

[1] 1 dead on the sixth day.

After one day of regeneration all the bulbs of gut present are practically in line with the central longitudinal axis of the body (Fig. 1). The anal opening (not a true anal opening, for it is completely surrounded by gut which forms part of the outside wall in these cases) is, therefore, terminal. On the second day, however, those pieces in 0.102M and weaker solutions have bulbs tipped at an angle so that the opening is not exactly terminal (Fig. 2). This change in the position of the bulbs does not occur as soon in 0.113M and 0.125M; it does appear, however, usually in all individuals by the fifth or sixth day. But few individuals show any evidence of this tipping in 0.14M. In this respect the individuals cited in Table I. under 0.136M were exceptional, since none happened to show evidence of new tissue. The underlying cause of this slight shifting of the position of the bulb is apparently the growth of tissue on one side of the wound region and not on the other. The region in which growth occurs is found, on microscopical examination, to be always ventral.

There is, then, from the macroscopical point of view, a graded effect on regeneration in *Lumbriculus* produced by a series of various concentrations of Ringer solution. In an approximately isotonic solution the majority of individuals show little evidence of the production of new tissue; except in rare cases those individuals which do produce sufficient new tissue to make it apparent do so only on the ventral side. In weaker solutions new tissue is produced in practicaly all cases. In dilutions but slightly hypotonic (0.08M to 0.13M) this process is confined to the ventral region in most instances. In most cases where new tissue is produced throughout the wound region, no bulb of gut is present. Occasionally, however, a very small bulb may be found at the end of a regenerating bud which is 1 or 2 mm. long. In solutions below 0.08M, on the other hand, practically every individual produces new tissue throughout the wound region and bulbs of gut are rare.

## Summary and Discussion.

In *Lumbriculus*, therefore, it would appear that an important factor in arousing the cells to regenerative activity is the dilution of the body fluids with water. When an individual is cut or

broken into two or more pieces, there are open wounds through which the body fluids may flow out and water may enter. This entrance of water is perhaps increased by the "writhing" and "crawling" movements of pieces immediately after they are cut. These movements would also extend the region of dilution a slight distance from the wound. The author (Sayles, '27) has reported that for mesoderm and intestine regenerative activity involves between 10 and 12 segments from the wound region. In the case of the hypodermis, which is commonly bathed on the outside by a hypotonic solution, activation occurs only in a restricted region near the wound. This limited activity of the hypodermis is due perhaps to the fact that the diluted fluids come in contract with its inner surface underneath the muscles which are pulled away near the wound. Farther away than that, however, the diluted body fluids probably never reach the hypodermal cells through the relatively thick layer of muscle cells.

While the hypotonicity of the water to the body fluid seems to be an activating factor in *Lumbriculus,* too general conclusions cannot be drawn from such results. In other fresh water forms this factor may be found to be of importance but in marine animals regeneration can certainly take place in a medium which is presumably of the same osmotic pressure as their body fluids. In these animals, however, greater regenerative activity may occur in slightly diluted rather than in normal sea-water. This has been reported by Goldfarb ('07, p. 353) in the hydroid, *Eudendrium,* in which "the maximum number of polyps regenerated does not occur in normal sea-water but in solutions diluted with about 20 per cent. of tap-water."

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# VARIATION OF HOOKS ON THE HIND WING OF THE HONEY BEE (*APIS MELLIFERA* L.).[1]

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The present paper represents partial results of a series of investigations carried on by the author since 1924 in the field of biometry of the honey bee (See Alpatov, 1–10). The material for this work has been collected partly during the author's work in the Zoölogical Museum of the Moscow University, and partly (material on American bees) during the summer of 1927 in the apicultural laboratory of the Agricultural College, Cornell University. The definite calculations and the preparation of the manuscript have been completed during the winter 1927–28 in the Institute for Biological Research. The author is glad to express his deep indebtedness to Professor Koshewnikov (Moscow), Professor E. F. Phillips (Cornell University), and Professor Raymond Pearl for their interest and help. The author also appreciated very much the help given by beekeepers of Russia and U. S.A. in collecting bees from different parts of both countries. Profeessor E. F. Phillips has also been so kind as to show the author the manuscript of his unpublished paper.

In spite of the fact that the beekeeper's literature contains a tremendous number of observations on differences in bee races, a scientific basis of racial studies in bees is practically absent, especially in comparison with racial and genetical studies on other domestic animals. The cause of this lies chiefly in certain peculiarities which characterize the honey bee. Firstly, the bees being fecundated in air do not allow us to control the mating and therefore to conduct exact genetical experiments. Secondly, it is more difficult to study the characteristics of such small animals as the honey bee than those of domestic mammals and birds. Only quite recent progress in artificial insemination of the queen

[1] From the Institute for Biological Research, Johns Hopkins University.

(Watson, '20) gives us the hope of being able to overcome the first of these obstacles.

The present author believes that a careful investigation of variation should be made before any attempts to study the heredity of the honey bee. In this direction the present paper brings evidence of the importance of a certain characteristic, namely, the number of the hooks, characterizing different biological groups in the limits of the species *Apis mellifera* L. Thanks to the modern investigations mostly of Russian scientists (Koschewnikov, Chochlov, Michailov, Alpatov, Alpatov and Tjunin) two very important facts in the field of variation of the honey bee have been discovered.

The first of them is the geographical regularity in the variation. The changes in tongue length of the worker bee is the most striking fact in the geographical variation of the honey bee. We are able to say that for countries with native bee population each locality is characterized by a definite tongue-length of bees inhabiting the given locality. Moreover, the change from one locality to another is regular and gradual. A general rule can be established; the more to the south, the longer the tongue length. Other body characteristics also show some regularities in geographical variation (Alpatov, 8). The author of the present paper believes that it is perfectly justifiable to compare the different "races" of the honey bee with geographical subspecies of wild animals.

Family variation is the second important fact which every investigator in the field of variability of social insects has to bear in mind. It was shown by several investigators, Thomson, Bell and Pearson (23, 24), Warren (25), Arnoldi (12), Z. G. Palenitschko (20), Alpatov and Tjunin (1) and Alpatov (3, 4, 6, 9, 10), that the variation of single families is smaller than the variation of the whole population. Therefore, in establishing racial characteristics we have to collect our material from as many families as possible.

Turning our attention to the literature devoted to the special question of hook variation we find only a small number of papers dealing with that particular subject. Professor Koschewnikov (19) was the first who introduced the number of hooks in the

taxonomy of the honey bee. E. B. Casteel and E. F. Phillips (14) without using biometrical methods, tried to solve the problem of comparative variability of drones and worker bees. Kellog's (17) data have also a very restricted value from the point of view of modern biometry. Wright, Lee and Pearson (27) then attempted, by recalculating Casteel's and Phillips' data, to draw some more definite conclusions. The most extensive work has been done by Bachmetjew (13). The conclusions of this author found just criticism in Koschewnokov's (18) and Ray-

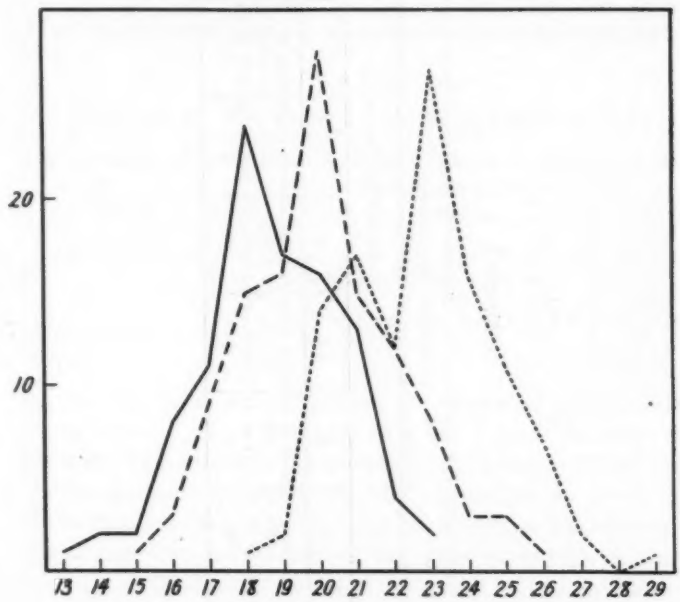


FIG. 1. Hook variation of 3 colonies of the Bulgarian drones.

mond Pearl's (21) papers and need not be mentioned further. Fortunately, Bachmetjew published in his paper all his numerous countings (about 2,500 bees were examined). His data have been worked out biometrically by the author of the present paper, and published in Russian (4). Professor Phillips did the same in the paper which is now in press. In this paper Professor Phillips turns his attention mostly to the individual variation in

## TABLE I.

CONSTANTS OF VARIATION CALCULATED FOR DRONES OF 10 COLONIES OF BULGARIAN BEES TAKEN FROM DIFFERENT APIARIES.
(Data from Bachmetjew.)

| | Number of the Colony. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| M | 21.56 ± .11 | 21.59 ± .14 | 22.18 ± .13 | 22.75 ± .13 | 21.16 ± .16 | 22.69 ± .14 | 22.13 ± .13 | 21.53 ± .13 | 18.68 ± .13 | 20.05 ± .14 |
| σ | 1.645 | 1.831 | 1.622 | 2.001 | 2.755 | 2.053 | 1.665 | 1.802 | 1.959 | 2.151 |
| C% | 7.63 ± .37 | 8.48 ± .44 | 7.31 ± .41 | 8.80 ± .40 | 13.02 ± .56 | 9.05 ± .44 | 7.52 ± .36 | 8.37 ± .41 | 10.49 ± .51 | 10.73 ± .48 |
| Number of cases | 99 | 83 | 71 | 110 | 127 | 100 | 100 | 93 | 100 | 114 |

the honey bee, and on that account his conclusions do not parallel those of the present paper.

The number of bees examined by the author of the present paper exceed three thousand—a number which has never been reached by previous investigators.

Table I. shows us the variation of Bulgarian drones belonging to different colonies. It is evident that the difference between the averages are in many cases more than five times larger than their probable errors. Fig. 1 represents 3 variation curves of the 4th, 9th and 10th colonies, proving the conclusion just made. Table II. shows the same for worker bees. It can be seen that in

TABLE II.

CONSTANTS FOR WORKERS OF 5 COLONIES OF BULGARIAN BEES (DATA FROM BACHMETJEW).

| | Number of the Colony. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| M | 21.60 ± .10 | 21.01 ± .09 | 21.76 ± .10 | 21.91 ± .12 | 21.11 ± .10 |
| $\sigma$ | 1.487 | 1.367 | 1.566 | 1.867 | 1.467 |
| C% | 6.88 ± .33 | 6.51 ± .31 | 7.20 ± .33 | 8.52 ± .41 | 6.95 ± .33 |
| Number of cases | 99 | 99 | 110 | 100 | 98 |

the last case the differences are not so pronounced as in the case of the drones. Fig. 2 compared with Fig. 1 gives the same impression. If we consider the coefficients of variation we find that for the drones they vary in the limits 7.52–13.02 per cent.; for the worker bees 6.88–8.52 per cent. It is obvious that the average variation of worker bees of the colony is smaller than the variation of the drones.

Are we justified in concluding that the drones are more variable than the worker bees? There is a certain weak point in such conclusions. We are not convinced that the method of collecting the material was safe enough to provide us with bees really representing the progeny of single queens—*i.e.*, members of one family. The proper way to get such a material would be to put a sealed brood in an incubator and collect the emerging bees. In collecting bees directly from the hive there is a danger of picking up bees belonging to the population of a neighbour hive. It

is known that the bees and especially the drones sometimes penetrate into neighboring hives. The only way to avoid this diffi-

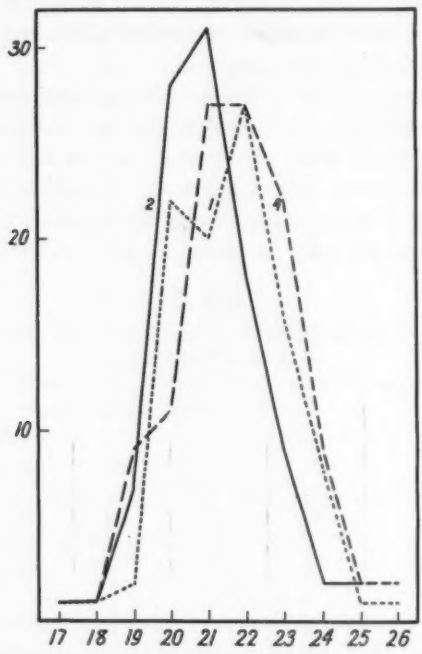


FIG. 2. Hook variation of 3 colonies of the Bulgarian worker bees.

culty is to calculate the coefficient of variation for the whole mass of bees. The results of such processes are shown in Table III. It can be seen that the variation of the worker bees belonging to

TABLE III.

BULGARIAN BEES IN DIFFERENT GROUPINGS.

| All Drones 1–10 Colonies. | Workers from 5 Colonies. | Queens from 10 localities in Bulgaria. | Drones from 1–5 Colonies. | Drones from 5–10 Colonies. |
|---|---|---|---|---|
| 21.39 ± .05 | 21.49 ± .05 | 18.46 ± .11 | 21.82 ± .07 | 20.98 ± .07 |
| 2.352 | 1.586 | 1.892 | 2.157 | 2.438 |
| 11.00 ± .17 | 7.38 ± .16 | 10.25 ± .42 | 9.89 ± .42 | 11.62 ± .25 |
| 997 | 507 | 139 | 490 | 507 |

the 5 colonies is lower than the variation of the two groups of drones each representing members of 5 colonies. The coefficients of variation calculated from our original material on worker bees are also in general lower than 8 per cent. Even for 1000 worker bees from Middle Russia taken from 106 colonies the coefficient of variation is only $8.539 \pm .129$, as can be seen from Table X. We believe that the present material permits us the definite conclusion of a larger variability of drones in respect to the number of hooks.

Table III. contains also data on variation of hooks in queens. Firstly, it is evident that the average number of hooks is far lower than in the drones and workers, which have practically the same averages. This conclusion is given here in statistical form for the first time, although G. A. Koschevnikov has already given a few analogous data. In respect of the coefficient of variation the queens are nearer to the drones than to the workers. A very incomplete material collected in Table IV. shows that Middle Russian, German and American black and yellow queens have also a much lower average number of hooks than the worker bees of the corresponding races.

TABLE IV.

NUMBER OF HOOKS OF DRONES AND QUEENS FROM DIFFERENT LOCALITIES.

| | Drones. | | | Queens. | | |
|---|---|---|---|---|---|---|
| | Moscow. | Kaluga (M. Russia). | N. Wodolaga (S. Russia). | Black-Ontario. | Italians. | Moscow and Darmstadt. |
| M..... | 20.72 | 20.22 ± .20 | 20.83 ± .26 | 16.25 | 18.00 | 18.67 ± .22 |
| C%..... | — | 9.76 ± .69 | 12.00 ± .87 | — | — | 9.06 ± .83 |
| No. of cases..... | 25 | 45 | 48 | 8 | 10 | 27 |

It is interesting to note that among the bees the relations of castes in respect of variation differ from those found in other social insects. It was shown (Palenitschenko, 20) that among wasps, termites and ants, the workers are more variable than the sexual forms—males and females. The worker caste among bees is therefore an exceptionally constant and standardized group of individuals.

Already in an earlier paper (4) some evidence has been brought

together to show that the bees of southern localities have a greater average number of hooks than the northern ones. In order to check that statement on a more solid basis, a special material has been collected from different parts of European Russia and the Caucasus. The map in Fig. 3 shows the localities which supplied a

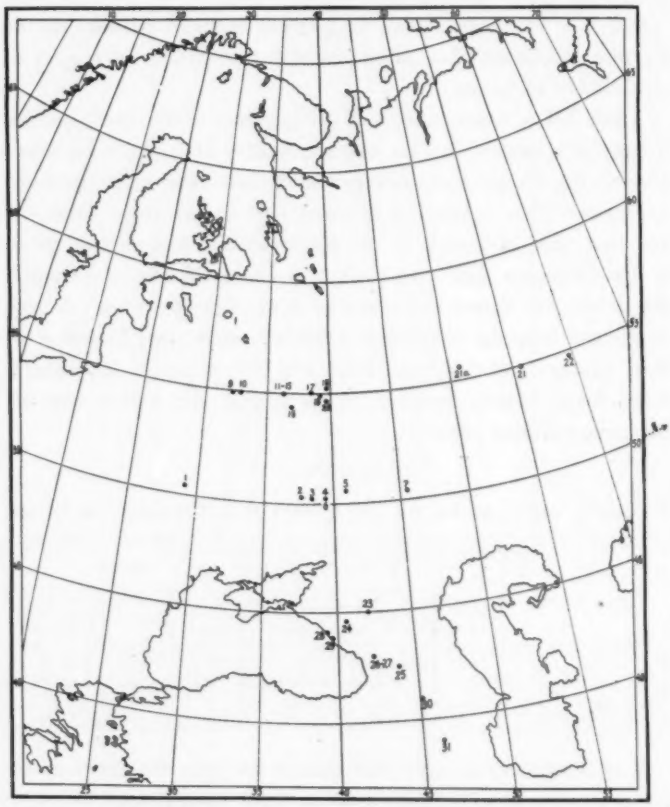

FIG. 3. Map of European Russia and Caucasus. The figures correspond to localities where the material has been collected.

corresponding material. The plain of European Russia is populated by the black variety of *Apis mellifera* L—*A. mellifera mellifera* L. An introduction of foreign blood, mostly of Italian queens, was according to certain statistical studies a compara-

tively rare phenomenon and could probably not produce any significant influence on the whole mass of the bee population of Russia (the number of hives in Russia according to certain estimations runs over 5,000,000). Tables V. and VI show the fre-

TABLE V.

FREQUENCY DISTRIBUTIONS AND CONSTANTS OF THE NUMBER OF HOOKS OF BEES FROM MIDDLE RUSSIA.

| No. of Hooks. | Localities. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17–18 | 20 | 19 | 20a | 21 | 22 |
| 29 | | | | | | | | | | | | | 1 | |
| 28 | | | | | | | | | | | | | | |
| 27 | 1 | | | | | | | | | 1 | | | | |
| 26 | 2 | | | | 1 | | | | | 0 | | 1 | 1 | |
| 25 | 1 | | 1 | 1 | 0 | | | 1 | 2 | 3 | | 2 | 2 | |
| 24 | 6 | | 1 | 3 | 1 | 2 | 1 | 1 | 3 | 0 | | 10 | 4 | 3 |
| 23 | 3 | 4 | 4 | 9 | 6 | 2 | 5 | 4 | 13 | 7 | 4 | 10 | 11 | 9 |
| 22 | 20 | 14 | 12 | 8 | 13 | 8 | 11 | 9 | 8 | 13 | 4 | 17 | 21 | 10 |
| 21 | 21 | 19 | 14 | 6 | 16 | 10 | 18 | 16 | 25 | 10 | 10 | 18 | 26 | 8 |
| 20 | 24 | 22 | 13 | 13 | 13 | 12 | 13 | 12 | 22 | 12 | 11 | 17 | 22 | 9 |
| 19 | 13 | 25 | 8 | 8 | 11 | 18 | 4 | 10 | 18 | 3 | 5 | 13 | 9 | 10 |
| 18 | 8 | 14 | 2 | 5 | 1 | 4 | 7 | 5 | 4 | 1 | 4 | 9 | 3 | 3 |
| 17 | 1 | 1 | 2 | 1 | | 4 | 1 | 2 | 2 | | 2 | 2 | | |
| 16 | | 1 | 2 | 1 | | 1 | 1 | | 3 | | | | | |
| No. of cases | 100 | 100 | 59 | 55 | 62 | 61 | 61 | 60 | 100 | 49 | 40 | 99 | 100 | 52 |
| No. of colonies | 7 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 18 | 6 | 4 | 10 | 9 | 6 |
| M | 20.86 ± .13 | 19.99 ± .10 | 20.56 ± .16 | 20.75 ± .18 | 20.92 ± .12 | 19.97 ± .15 | 20.56 ± .14 | 20.48 ± .14 | 20.58 ± .12 | 21.48 ± .16 | 20.28 ± .00 | 21.04 ± .15 | 21.27 ± .12 | 20.98 ± .16 |
| C% | 8.97 ± .43 | 7.47 ± .35 | 8.64 ± .54 | 9.45 ± .61 | 6.77 ± .41 | 8.51 ± .52 | 7.77 ± .47 | 8.04 ± .50 | 8.04 ± .43 | 7.97 ± .54 | 7.64 ± .58 | 9.11 ± .44 | 8.14 ± .39 | 8.08 ± .53 |

quency distribution for different localities, number of colonies and corresponding biometrical characteristics. Table X. gives summarized frequencies for the bees distributed in Middle Russia at the level of 55° of N. latitude and in South Russia, 50° N. latitude. The difference between the averages is 8.85 times larger than the probable error. We may conclude, therefore, with a high degree of certainty, that there is an increase in the average

## TABLE VI.

FREQUENCY DISTRIBUTION AND CONSTANTS OF THE NUMBER OF HOOKS OF BEES FROM SOUTH RUSSIA.

| No. of Hooks. | Localities. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2–3 | 4 | 5 | 6 | 7 | 8 |
| 26 | 1 | | 1 | 1 | 1 | | |
| 25 | 3 | 1 | 0 | 2 | 2 | 3 | |
| 24 | 4 | 7 | 2 | 5 | 4 | 8 | 2 |
| 23 | 10 | 12 | 6 | 7 | 6 | 4 | 7 |
| 22 | 17 | 18 | 11 | 10 | 12 | 13 | 10 |
| 21 | 10 | 19 | 8 | 11 | 24 | 8 | 20 |
| 20 | 10 | 22 | 6 | 12 | 21 | 8 | 14 |
| 19 | 5 | 15 | 9 | 5 | 15 | 4 | 4 |
| 18 | 3 | 3 | 4 | 6 | 7 | 1 | 3 |
| 17 | 1 | 3 | 1 | 1 | | 1 | |
| No. of cases | 64 | 100 | 48 | 60 | 92 | 50 | 60 |
| No. of colonies | 6 | 18 | 10 | 6 | 9 | 5 | 6 |
| M | 21.52 ± .16 | 20.94 ± .12 | 20.88 ± .18 | 21.13 ± .18 | 20.75 ± .12 | 21.68 ± .18 | 20.98 ± .12 |
| C% | 8.59 ± .51 | 8.29 ± .40 | 8.98 ± .62 | 9.53 ± .59 | 8.21 ± .41 | 8.63 ± .58 | 6.60 ± .41 |

number of hooks in the southern direction even between groups of bees in comparatively closely situated localities. Turning our attention to the Caucasus (Fig. 5) we must say that the situation here is more complicated than in the plain of European Russia. Zoögeographically, the Caucasus is divided into several sharply limited provinces, each of them with peculiarities in the composition and the origin of the organic life. The Caucasus bees are also not homogenous. The best characterized is the gray Caucasian mountain bee *Apis mellifera caucasica* Gorbatschev and the yellow Transcaucasian so-called Persian bee. This bee was first recognized as an independent species by Pallas; although he did describe the Caucasian bee he has never published his manuscript. The specimen with the original label is preserved in the Berlin Zoölogical Museum and was briefly described by

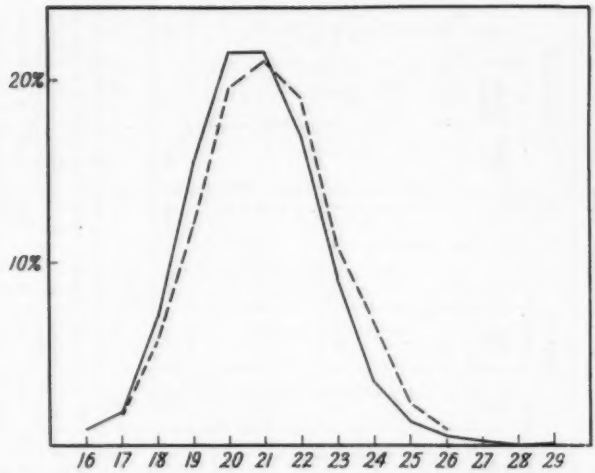


FIG. 4. The continuous curve represents the variation of hooks of bees from Middle Russia. The dotted, is based on material from South Russia. The frequencies are expressed in percents.

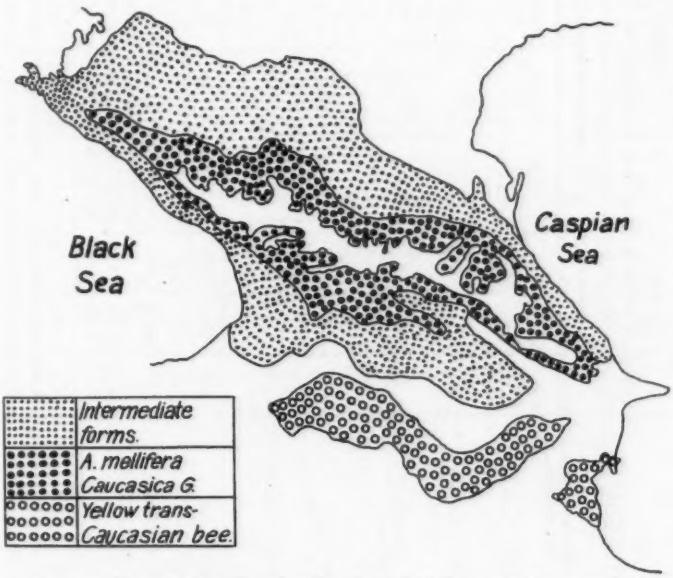


FIG. 5. Map of Caucasus showing the distribution of fifteen variations of bees (after A. Gorbatschev).

## TABLE VII.

FREQUENCY DISTRIBUTIONS AND CONSTANTS OF THE NUMBER OF HOOKS OF CAUCASIAN BEES.

| No. of Hooks. | N. Caucasus. | | | Caucasus Mountains. | | | | | Abchasia. | | | | | Transcaucasia. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Stavropol 23. | Maikop 24. | Σ. | Tiflis 25. | | Svanetia 26. | Kutais 27. | Σ. | I/28. | II/28. | III/28. | 29. | Σ. | Migri 30. | Delidjan 31. | Σ. |
| 27 | | | | | | | | | | | | | | 1 | | 1 |
| 26 | | | | | | | 1 | 1 | | | | | | 1 | | 1 |
| 25 | 1 | 2 | 3 | 1 | 3 | | 0 | 4 | 1 | 2 | | | 3 | 0 | | 0 |
| 24 | 2 | 1 | 3 | 0 | 3 | 2 | 2 | 7 | 0 | 1 | 2 | 4 | 7 | 2 | 3 | 5 |
| 23 | 10 | 8 | 18 | 6 | 2 | 10 | 2 | 20 | 9 | 4 | 9 | 8 | 30 | 4 | 7 | 11 |
| 22 | 24 | 19 | 43 | 13 | 5 | 10 | 9 | 37 | 6 | 9 | 9 | 10 | 34 | 8 | 14 | 22 |
| 21 | 40 | 24 | 64 | 9 | 6 | 6 | 9 | 30 | 9 | 10 | 19 | 17 | 55 | 4 | 8 | 12 |
| 20 | 43 | 30 | 73 | 12 | 4 | 4 | 4 | 24 | 8 | 8 | 12 | 13 | 41 | 8 | 6 | 14 |
| 19 | 24 | 12 | 36 | 7 | 1 | 2 | 5 | 15 | 9 | 5 | 5 | 8 | 27 | 1 | 1 | 2 |
| 18 | 6 | 3 | 9 | 2 | | 2 | | 4 | 3 | 2 | 2 | 5 | 12 | | 1 | 1 |
| 17 | 5 | 1 | 6 | | | | | | | | 2 | 0 | 2 | | | |
| 16 | 1 | | 1 | | | | | | | | | 0 | 0 | | | |
| 15 | | | | | | | | | | | | 0 | 0 | | | |
| 14 | | | | | | | | | | | | 1 | 1 | | | |
| No. of cases | 156 | 100 | 256 | 50 | 24 | 36 | 32 | 142 | 45 | 41 | 60 | 66 | 212 | 29 | 40 | 69 |
| No. of colonies | ? | many | — | 8 | 7 | several | ? | — | 9 | 10 | 12 | about 10 | — | 3 | 4 | 7 |
| M | 20.47 ±.11 | 20.79 ±.10 | 20.61 ±.06 | 20.94 ±.14 | 22.00 ±.24 | 21.61 ±.17 | 21.31 ±.19 | 21.33 ±.10 | 20.84 ±.17 | 21.10 ±.17 | 20.95 ±.14 | 20.82 ±.15 | 20.91 ±.08 | 21.79 ±.23 | 21.65 ±.14 | 21.71 ±.13 |
| C% | 7.31 ±.28 | 6.94 ±.33 | 7.14 ±.21 | 7.11 ±.48 | 7.87 ±.77 | 7.19 ±.57 | 7.49 ±.63 | 7.91 ±.32 | 8.14 ±.58 | 7.18 ±.58 | 7.42 ±.46 | 8.73 ±.52 | 7.93 ±.26 | 8.42 ±.75 | 6.16 ±.46 | 7.19 ±.41 |

## Table VIII.

Differences between the Average Number of Hooks in Different Groups of Bees.

| | | 55°. | 50°. | North Caucasus. | Abchasia. | Caucasus Mountains. | Trans-caucasia. | Italy. | American Italians. | American Black Bees. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 20.70 ± .04 | 21.08 ± .06 | 20.61 ± .06 | 20.91 ± .08 | 21.33 ± .10 | 21.71 ± .13 | 21.51 ± .08 | 21.00 ± .06 | 19.63 ± .09 |
| 55° | 20.70 ± .04 | 0 | | | | | | | | |
| 50° | 21.08 ± .06 | 0.62 ± .07<br>R = 8.85 | 0 | | | | | | | |
| North Caucasus | 20.61 ± .06 | 0.09 ± .07<br>R = 1.29 | 0.47 ± .08<br>R = 5.88 | 0 | | | | | | |
| Abchasia | 20.91 ± .08 | 0.21 ± .09<br>R = 2.3 | 0.17 ± .10<br>R = 1.70 | 0.30 ± .10<br>R = 3.00 | 0 | | | | | |
| Caucasus Mountains | 21.33 ± .10 | 0.63 ± .11<br>R = 5.73 | 0.25 ± .12<br>R = 2.08 | 7.2 ± .12<br>R = 6.00 | 0.42 ± .13<br>R = 3.23 | 0 | | | | |
| Transcaucasia | 21.71 ± .13 | 1.7 ± .14<br>R = 7.22 | 0.63 ± .14<br>R = 4.5 | 1.10 ± .14<br>R = 7.86 | 0.80 ± .15<br>R = 5.33 | | 0 | | | |
| Italy | 21.51 ± .08 | 0.81 ± .09<br>R = 9.00 | 0.43 ± .10<br>R = 4.3 | 9.0 ± .10<br>R = 9.0 | 0.60 ± .11<br>R = 5.45 | 0.18 ± .13<br>R = 1.38 | 0.20 ± .15<br>R = 1.33 | 0 | | |
| American Italians | 21.00 ± .06 | 0.30 ± .07<br>R = 4.29 | 0.08 ± .08<br>R = 1.00 | 0.39 ± .08<br>R = 4.87 | 0.09 ± .10<br>R = 0.09 | 0.33 ± .12<br>R = 2.75 | 0.71 ± .14<br>R = 5.07 | 0.51 ± .09<br>R = 5.67 | 0 | |
| American black bees | 19.63 ± .09 | 1.07 ± .10<br>R = 10.7 | 1.45 ± .11<br>R = 13.2 | 0.98 ± .11<br>R = 8.90 | 1.28 ± .12<br>R = 10.66 | 1.70 ± .13<br>R = 13.08 | 2.08 ± .16<br>R = 13.00 | 1.88 ± .12<br>R = 15.66 | 1.37 ± .11<br>R = 12.45 | 0 |

Gerstäcker (15). The author of the present article was able, thanks to the courtesy of the curator of the collection of Hymenoptera of the Berlin Zoölogical Museum, Professor Dr. H. Bischoff, to examine Pallas's specimen as well as his manuscripts. Pallas gives in his manuscript the following indication about the origin of his Caucasian yellow bee: " Ad Caucasum lecta, itemque ex Hyrcania transmissa fuit." The small size and pronounced yellow coloration of the specimens preserved in the Berlin Museum permit us to conclude that Pallas and Gerstäcker described under the name *Apis remipes*, the Transcaucasian Persian bee, but not the north Caucasian darker bees.

Some peculiarities—for instance a much longer tongue—distinguish *Apis mellifera remipes* Gerstäcker (not Pallas) from the Italian bee *Apis mellifera ligustica Sp.* It is therefore not correct to identify the *A. m. remipes* with the Italian bees (*Apis ligustica*) as it has been done by G. A. Koschewnikov. According to Gorbatschev (see the map in Fig. 5 taken from his article (16) ) the prairies and hills of the northern Caucasus and the valleys of Transcaucasia are populated by a bee of intermediate type—hybrids in his interpretation. We united our material into four groups: (*a*) N. caucasus bees, (*b*) bees from four apiaries near the coast of the Black Sea—Abchasian, (*c*) gray Caucasian mountain bees (*A. mellifera caucasica* Gorb.) and (*d*) yellow Transcaucasian bees (*Apis mellifera remipes* Gerst.). Table VII. shows the frequency distributions and Table VIII. gives us material for estimating the importance of our differences. The *Apis m. caucasica* and *remipes* show a pronounced higher number of hooks than bees of South Russia. Of course such a comparatively limited number of colonies from N. Caucasus does not permit us to draw a perfectly definite conclusion. It is interesting to note that the gray Caucasian bees imported to the United States (see Table IX.) gave also a high average of the number of hooks.

Table IX. gives us some data on other European races of bees. The Italian bees from Italy are characterized by a high number of hooks. It can be seen that the progeny of Italian queens imported from Italy to N. Caucasus shows also a high number of hooks. The German black bees, according to our recalculations

## TABLE IX.

BEES FROM DIFFERENT LOCALITIES IN EUROPE AND U. S. A.

| No. of Hooks. | Caucasians from Colorado. | Italians from Italy. | Black Bees. | | | | Italians from Caucasus. | German Black Bees (Armbruster). | Italians in U. S. A. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Florida. | N. Carolina. | Ontario. | Total. | | | Alabama 1. | Kentucky. | Alabama 2. | Ohio 1. | Ohio 2. | New York. | Ithaca, N. Y. | Pennsylvania. |
| 26 | 1 | | | | | | 1 | 0 | | | | | | | | |
| 25 | — | 2 | | | | | 1 | 4 | | | 1 | | | 1 | 1 | 1 |
| 24 | 2 | 13 | | 4 | | 4 | 5 | 9 | 3 | 3 | 3 | 2 | 6 | 2 | 2 | — |
| 23 | 10 | 26 | | 3 | | 3 | 9 | 27 | 5 | 7 | 8 | 8 | 10 | 1 | 4 | 3 |
| 22 | 11 | 32 | 5 | 9 | 1 | 15 | 13 | 37 | 7 | 11 | 9 | 14 | 7 | 10 | 12 | 14 |
| 21 | 19 | 43 | 5 | 9 | 6 | 20 | 10 | 49 | 16 | 14 | 14 | 10 | 12 | 15 | 18 | 11 |
| 20 | 9 | 19 | 11 | 11 | 11 | 33 | 6 | 55 | 11 | 3 | 7 | 8 | 9 | 10 | 5 | 10 |
| 19 | 4 | 10 | 16 | 8 | 17 | 41 | 4 | 28 | 3 | 8 | 3 | 4 | 4 | 8 | 5 | 8 |
| 18 | 4 | 4 | 9 | 7 | 10 | 26 | 0 | 14 | 3 | 4 | 5 | 4 | 2 | 2 | 3 | 4 |
| 17 | | 1 | 4 | 2 | 3 | 9 | 1 | 5 | 2 | | | | | 1 | 2 | 1 |
| 16 | | | | 1 | 2 | 3 | | 1 | | | | | | | | |
| No. of cases | 60 | 150 | 50 | 54 | 50 | 154 | 50 | 230 | 50 | 50 | 50 | 50 | 50 | 50 | 52 | 52 |
| No. of colonies | 10 | 10 | 10 | 10 | 10 | 30 | 7 | 7 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| M | 21.22 ±.14 | 21.51 ±.08 | 19.38 ±.13 | 20.37 ±.18 | 19.08 ±.12 | 19.63 ±.09 | 21.74 ±.21 | 20.84 ±.09 | 20.84 ±.16 | 21.06 ±.16 | 21.20 ±.15 | 21.16 ±.14 | 21.44 ±.16 | 20.72 ±.15 | 20.96 ±.16 | 20.57 ±.15 |
| C% | 7.46 ±.46 | 7.12 ±.28 | 7.07 ±.48 | 9.42 ±.61 | 6.78 ±.46 | 8.45 ±.32 | 7.51 ±.51 | 8.57 ±.27 | 7.87 ±.53 | 7.85 ±.53 | 7.40 ±.50 | 6.70 ±.46 | 7.64 ±.52 | 7.48 ±.50 | 8.02 ±.53 | 7.74 ±.51 |

TABLE X.

| No. of Hooks. | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ♂ from 10 Colonies Bulgaria. | | ♀ from 5 Colonies Bulgaria. | | ♀ Middle Russian Bees. | | ♀ South Russian Bees. | | ♀ Italian Bees in U. S. A. | | ♂ from 1 Colony (Data from Phillips). | |
| | Observed. | Calculated. | Observed. | Calculated. | Observed. | Calculated. | Observed. | Calculated. | Observed. | Calculated. | Observed. | Calculated. |
| 29 | 2 | .81 | | | 1 | 0.20 | | | | | | |
| 28 | 4 | 2.69 | | | — | 0.52 | | | | | | |
| 27 | 9 | 9.34 | | | 2 | 1.40 | | | | | 2 | 1.64 |
| 26 | 21 | 24.01 | 5 | 2.14 | 5 | 3.99 | 4 | 2.32 | | | 8 | 6.91 |
| 25 | 52 | 51.34 | 12 | 9.68 | 13 | 11.60 | 11 | 9.41 | 4 | 4.83 | 21 | 21.52 |
| 24 | 84 | 91.22 | 34 | 36.12 | 35 | 32.93 | 32 | 27.80 | 21 | 17.60 | 54 | 49.74 |
| 23 | 149 | 134.73 | 81 | 81.13 | 91 | 84.15 | 52 | 59.74 | 46 | 46.82 | 82 | 85.15 |
| 22 | 154 | 165.41 | 112 | 122.08 | 168 | 171.39 | 91 | 93.65 | 84 | 82.77 | 104 | 107.93 |
| 21 | 183 | 168.76 | 121 | 122.68 | 217 | 243.76 | 100 | 106.93 | 110 | 100.00 | 98 | 101.40 |
| 20 | 142 | 143.07 | 97 | 82.60 | 215 | 222.94 | 93 | 88.87 | 63 | 82.57 | 74 | 70.61 |
| 19 | 87 | 100.92 | 33 | 37.21 | 156 | 134.34 | 57 | 53.86 | 43 | 46.65 | 38 | 36.40 |
| 18 | 61 | 59.05 | 8 | 11.24 | 70 | 59.20 | 27 | 23.76 | 27 | 18.00 | 15 | 13.93 |
| 17 | 31 | 28.75 | 4 | 2.26 | 18 | 21.95 | 7 | 7.65 | 6 | 4.75 | 3 | 3.94 |
| 16 | 11 | 11.63 | | | 9 | 7.60 | | | | | 1 | 0.83 |
| 15 | 4 | 3.91 | | | | | | | | | | |
| 14 | 2 | 1.09 | | | | | | | | | | |
| 13 | 1 | 0.25 | | | | | | | | | | |
| N | 997 | 996.98 | 507 | 507.1 | 1,000 | 995.97 | 474 | 473.99 | 404 | 403.99 | 500 | 500.00 |
| M | 21.392 ± .050 | | 21.485 ± .047 | | 20.698 ± .038 | | 21.082 ± .055 | | 21.003 ± .054 | | 21.708 ± .055 | |
| σ | 2.32525 ± .03511 | | 1.57795 ± .03342 | | 1.76733 ± .02665 | | 1.77672 ± .03893 | | 1.62084 ± .03846 | | 1.82631 ± .03896 | |

TABLE X. (*Continued.*)

| No. of Hooks. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| | ♂ from 10 Colonies Bulgaria. | ♀ from 5 Colonies Bulgaria. | ♀ Middle Russian Bees. | ♀ South Russian Bees. | ♀ Italian Bees in U. S. A. | ♂ from 1 Colony (Data from Phillips). |
| C%..... | 10.870 ± .164[1] | 7.344 ± .156 | 8.539 ± .129 | 8.428 ± .185 | 7.717 ± .183 | 8.413 ± .179 |
| $\beta_1$..... | 0.001585 ± .000723 | 0.019907 ± .010763 | 0.078324 ± .026122[2] | 0.031206 ± .014291 | −0.021689 ± .010207 | 0.000795 ± .000373 |
| Ratio $\frac{\beta_1}{\text{P.E.}}$ | 2.192 | 1.850 | 2.998 | 2.184 | 2.124 | 2.131 |
| $\beta_2$..... | 3.247403 ± .146382 | 3.045287 ± .165798 | 4.194584 ± .104515[2] | 2.719259 ± .107575 | 2.685580 ± .108751 | 2.817037 ± .110263 |
| Ratio $\frac{\beta_2}{\text{P.E.}}$ | 1.690 | 0.273 | 11.429 | 2.610 | 2.89 | 1.708 |
| K..... | 0.002423 | 0.486326 | 0.027443 | −0.037931 | 0.023560 | −0.001621 |
| Type.... | Normal curve | ? | VII | Normal curve | Normal curve | Normal curve |
| Goodness of fit... | .7326 | .3131 | .2602 | .8775 | .1819 | .9977 |

[1] Calculated from the abbreviated formula like for all the other C. of V. Equation of the curve of the ♀ (Middle Russia, type VII)

$$y = 248.833 \quad 1 + \left(\frac{x^2}{21.935047}\right)^{-4.6118}.$$

[2] Calculated for normal curve of errors.

of Armbruster's data, give a number which corresponds to that of the Middle Russian ones.

Summing up now our whole material on European races we may say that there is much evidence for an assumption of a high number of hooks in southern races in comparison with northern ones. The Bulgarian group of bees also supports this conclusion. It would be interesting to test this rule on other castes of bee colonies. Unfortunately our material on drones from Russia is very small (see Table IV.), although it can be seen that the Middle

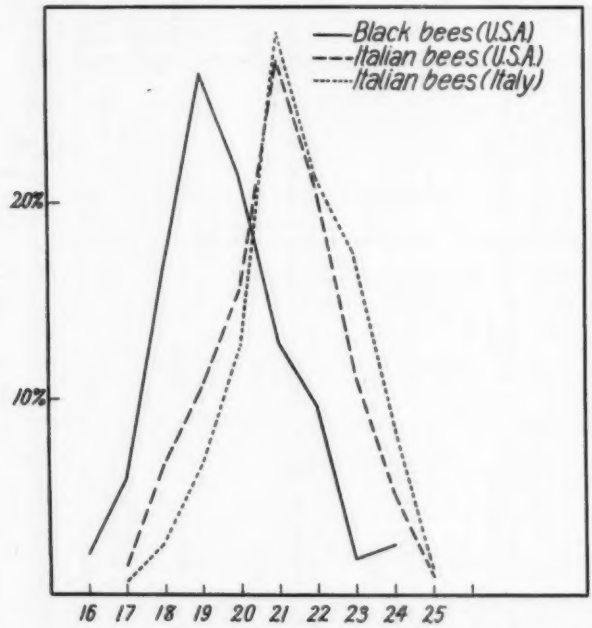


FIG. 6. Curves of variation of hooks. Continuous line—black bees in U. S. A.; dotted line—Italian bees from Italy; dash line—Italians in U. S. A.

Russian and even South Russian drones have a smaller average number of hooks than the Bulgarian ones. It would not be wise to draw any conclusions about the geographical differences in queens based on such a small number of cases. We have to add that Middle Russian and South Russian drones give the high degree of variation (C%) usual for drones.

It is well known that at the time of the discovery of the New World, America had no native bees. The first bees imported to this country came, according to historical data, from Holland and England and belonged to the common black bees *A. mellifera mellifera* L. About the middle of the last century the American beekeepers began to prefer for cultivation the yellow Italian bee, which is now the dominant race in this country. Thanks to the help of many beekeepers I have succeeded in examining, from a considerable number of apiaries, Italian bees of different degrees

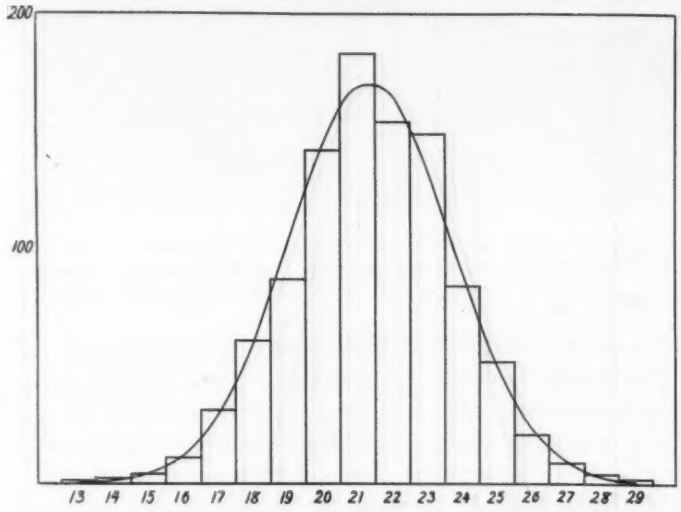


FIG. 7. Frequency polygon and fitted curve of the variation of the hooks of the Bulgarian drones.

of development of yellow color as well as pure black bees. Tables IX. and X. show us the variation of bees acclimatized to the United States. Firstly, we have to note the great difference in the number of hooks of black and yellow American bees, secondly, a little lower number of hooks of Americanized Italian bees than that of true Italians reared either in Italy or from Italian queens imported directly from that country. This is illustrated by curves on Fig. 6. The very low average number of American black bees as compared with our material discussed

above give us the right to suppose a general decrease of the number of hooks in the United States as compared with Europe, both in black and yellow bees. Further investigations need to be made with special attention to the problem of influence of acclimatization upon physical characteristics in the honey bee.

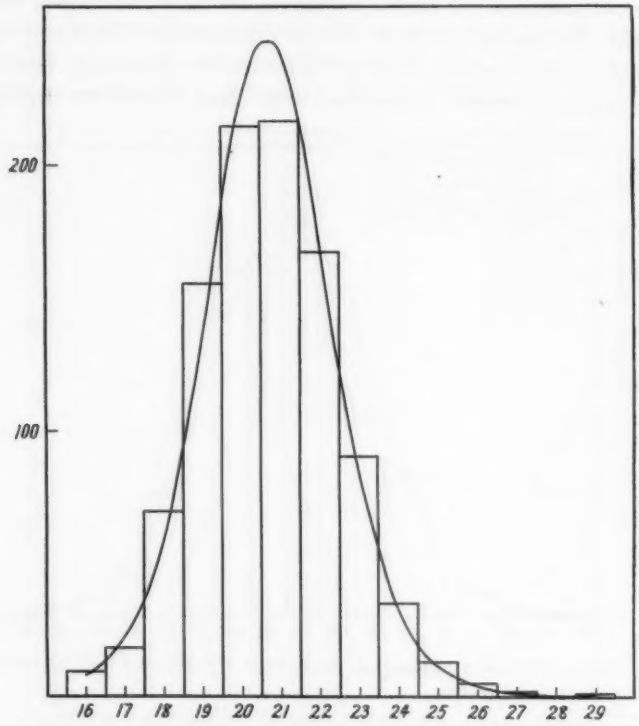


FIG. 8. Frequency polygon and fitted curve of variation of the worker bees from Middle Russia.

Our comparatively large material gave us the possibility of determining the character of the frequency distributions. The results are given in Table X. All distributions are symmetrical and only one shows a deviation from the normal distribution. A curve of type VII. was chosen to fit this distribution. The distribution for the Bulgarian worker bees being symmetrical and normal in regard to the $\beta_2$ gave a very high value of the criterion,

which leads us to the curve of type IV. It was not possible to calculate the probable errors of the criterion, $\beta_1$ and $\beta_2$ being too close to those characterizing the normal curve of error. Therefore a normal curve was used for fitting. We used for calculating the ordinates of the normal curve from Pearson's "Tables for Statisticians and Biometricians." Figs. 7 and 8 show two of our curve fittings.

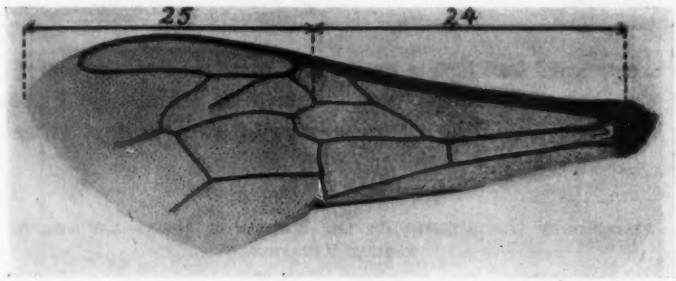


FIG. 9. Measurements on the wing. The wing shows the intercubitus vein not developed. (Microphotograph taken in the Art Department of the Institute for Biological Research, by Mr. Johansen.)

During the author's residence at Cornell University an attempt was made to study the influence of undernourishment of larvæ upon the characteristics of adult bees. The experiment consisted in putting the unsealed brood in an incubator running at 34.5° C. The brood was taken from a comb approximately one day before normal sealing. On the following day the cells situated in the neighborhood of the place from which a piece of comb had been taken the day before were already sealed by bees and also put in the incubator in order to provide us with control insects. Bees normally developed in hives were also collected from the frame of the hive which gave us material for the experiment. The pieces of comb with unsealed larvæ put in the incubator were covered with pieces of artificial comb foundation in substitution for the natural capping bees. The larvæ wove cocoons as usual and the emerging bees were collected in alcohol. The bees emerging from the unsealed brood evidently suffered from a certain underfeeding in comparison with control bees. Table XI. shows that the

TABLE XI.

CONSTANTS OF WING MEASUREMENTS OF CONTROL AND UNDERFED (IN LARVAL STAGE) BEES IN MM.

| | Proximal Length of Wing (Meas. N 24). | Distal Length of Wing (Meas. N 25). | No. of Cases. |
|---|---|---|---|
| Experimental (underfed) bees..... | 4.525 ± .021 | 4.192 ± .023 | 46 |
| Control bees..... | 4.696 ± .010 | 4.353 ± .012 | 31 |

experimental bees have a smaller size of wings than the controls. The characteristics have been measured, as is shown in Fig. 9. Table XII. shows the average number of hooks in three groups.

TABLE XII.

INFLUENCE OF UNDERFEEDING ON THE NUMBER OF HOOKS AND THE ABNORMAL VENATION.

| Character. | M ± P.E. | C%±P.E. | Percentage of Wing with Abnormal Vein. | Number of Specimens. |
|---|---|---|---|---|
| 1. Bees taken from the hive... | 20.77 ± .16 | 7.34 ± .56 | 0.00 — | 39 |
| 2. Bees reared in the incubator from brood normally fed.. | 20.60 ± .15 | 7.33 ± .52 | 4.44 ± 1.37 | 45 |
| 3. Bees reared in the incubator from underfed brood..... | 19.71 ± .09 | 7.39 ± .33 | 19.82 ± 2.50 | 116 |
| Diff. 1–3..... | 1.06 ± .18<br>R = 5.89 | — | 19.82 ± 2.50<br>R = 7.93 | |
| Diff. 2–3..... | 0.89 ± .17<br>R = 5.24 | — | 15.38 ± 2.85<br>R = 5.40 | |

It can be seen that the underfed bees have a smaller number of hooks than the control bees reared from the sealed brood and taken directly from the hive. The same is expressed in graphical form on curves of the Fig. 10. The experimental bees showed a quite peculiar type of abnormality in the venation of the first pair of wings. The abnormality consists in the incomplete development of the second intercubitus vein. The percentage expression of this abnormality in our three groups shows that the ab-

normality occurs also in bees reared in the incubator from normally sealed broods but reaches a very high grade of development in undernourished bees. The abnormalities in insect wings have been many times the subject of careful morphological studies. Our experiment opens a way for studying this problem by means of the experimental method. It is worth while to note that in geographical races the southern bees, being usually smaller than

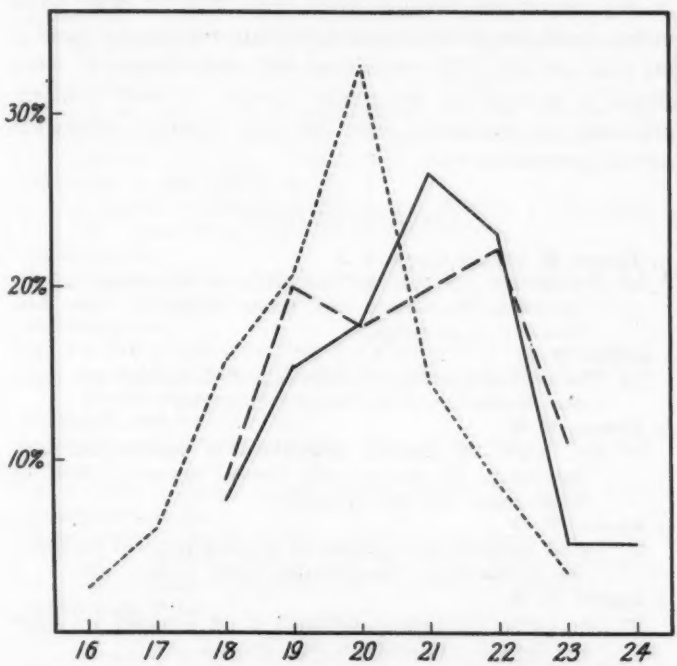


FIG. 10. Continuous line—bees from the hives which gave larvæ for the experiment with underfeeding. Dotted line—variation of hooks in the group of bees emerged from underfed larvæ. Dashed line—control bees developed from normally fed larvæ and emerged in the incubator together with underfed bees.

the northern one (Alpatov, 8), at the same time show an increase in the number of hooks. Our experimental bees gave the contrary relation. Therefore it is not possible to explain the smaller number of hooks of the northern bees by the assumption of an underfeeding of larvæ.

SUMMARY.

The data presented in this paper show that the average number of hooks in the honey bee is a characteristic which is differently developed among single colonies, sexes, castes, and races. As a general rule the southern races have a large number of hooks in worker bees and probably in drones. The queens and drones are more variable in regard to this character than the worker bees. In this respect the relations differ from those in other social insects (ants, wasps and termites), where the asexual caste is the most variable. The experiment with underfeeding of larvæ showed a decrease of the average number of hooks and the producing of specimens with defective venation—incomplete second intercubitus vein.

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